This number contains Indexes and completes Volume 63.

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15648

Electron Micrographs of the Agent of Feline Pneumonitis (Baker).

GEOFFREY RAKE, HELEN RAKE, DOROTHY HAMRE, AND VINCENT GROUPÉ.
From the Squibb Institute for Medical Research, New Brunswick, N.J.

The morphology of several viruses and rickettsiae has been studied with the aid of the electron microscope but no studies on any member of the psittacosis-lymphogranuloma-trachoma group have been published. Although the data presented here deal only with the agent of feline pneumonitis, similar micrographs have been obtained with agents of lymphogranuloma venereum, human pneumonitis* (S.F.) and psittacosis.*

The material for study was prepared from 10% or 20% suspensions of heavily infected yolk sacs which were shaken in Ringer's without beads for 20 minutes to liberate the agent from infected cells. From then on the procedure varied but the following gave the cleanest preparations. The supernate from shaking was twice centrifuged for 15 minutes at 2000 and at 2400 r.p.m. The agent was then sedimented twice at 15,000 r.p.m. for one hour and resuspended in Ringer's solution. After a third centrifugation at 15,000 r.p.m. for one hour the sediment was finally resuspended in the original volume of distilled water. This suspension was clarified finally at 2000 r.p.m. for 15 minutes and the supernate dried on collodion membranes in the usual manner.

As shown by the illustrations, the characteristic picture obtained is one in which a central more opaque mass is surrounded by a less opaque circular limiting membrane.

* We wish to thank Dr. Francis Gordon for supplying suspensions of these two agents which had been inactivated by ultraviolet irradiation.

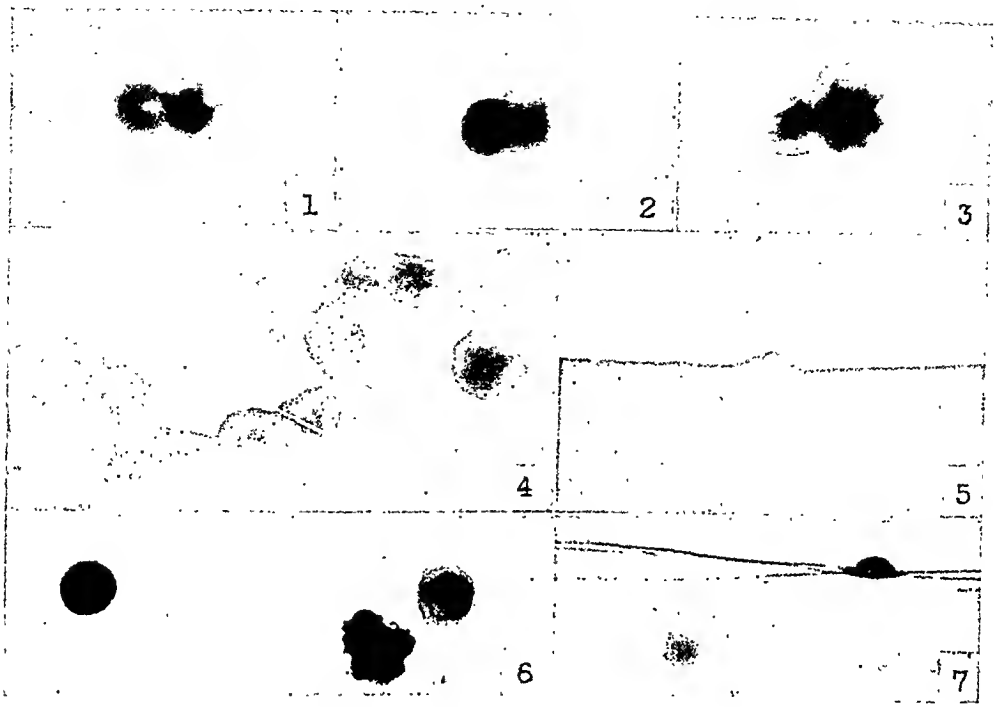

FIG. 1.
The elementary bodies of Feline Pneumonitis. Magnification 20,700 ×.

This appearance resembles closely that described for the rickettsiae[1-3] and certain bacteria,[4] less closely that shown by vaccinia[5,6] and not at all that of the smaller viruses. In the case of feline pneumonitis, the central substance is quite irregular internally as well as in outline (Fig. 1-4). The appearance suggests the irregularities of a wrinkled pea flattened on one side and in a few instances in which side views have been obtained due to rupturing of the collodion membranes, this opinion was confirmed (Fig. 5). In a very few instances dark bodies of uniform density are seen without any limiting membrane. It is not certain that these represent the bodies of the agent but since they are of the same general size and density as the central masses they may be undistorted bodies or bodies in which the substance of the limiting membrane has been lost (Fig. 2, 6). In some bodies showing the limiting membrane there was little distortion of the central mass. In side view such bodies appear like derby hats (Fig. 7).

The majority (51%) of the elementary bodies appear by direct measurement of electron micrographs to be between 440 and 490 mμ in diameter. The median diameter is 465 mμ, the average 455 mμ, and the range from 350 to 580 mμ. Such lack of uniformity is not seen with the smaller viruses. It seems probable that these diameters for the elementary bodies are larger than those existing in the viable forms. The flattened wrinkled pea form noted above suggests that the bodies resemble sacs filled with jelly which, settling down on the collodion membranes, lose their spherical shape and increase

[1] Plotz, H., Smadel, J. E., Anderson, T. F., and Chambers, L. A., *J. Exp. Med.*, 1943, **77**, 355.

[2] Weiss, L. J., *J. Immunol.*, 1943, **47**, 353.

[3] Shepard, C. C., and Wyckoff, R. W. G., *Pub. Health Rep.*, 1946, **61**, 761.

[4] Mudd, S., and Anderson, T. F., *J. Am. Med. Assn.*, 1944, **126**, 561.

[5] Green, R. H., Anderson, T. F., and Smadel, J. E., *J. Exp. Med.*, 1942, **75**, 651.

[6] Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 259.

in diameter. Larger bodies, such as might correspond to initial bodies, are rare although at least one 770 mμ in diameter has been noted. There are two possible reasons for the paucity of initial bodies. They may be largely discarded with the low speed sediment or they may fragment, at least to the extent of losing their capsular material, in the process of repeated differential centrifugation. There is evidence that both occur. Thus examination with the light microscope of low speed supernates of preparations, originally rich in initial bodies, show complete disappearance or marked decrease in numbers—to between 1% and 2%. Such supernates are then further treated, by repeated high speed sedimentation and resuspension, and those showing any initial bodies suffer a further 90% decrease.

Pairs, chains and groups of elementary bodies are to be found (Figs. 1-4). Fairly dense intercellular bridges stretching between bodies are not infrequent and may be enclosed by limiting membranes (Fig. 4). The picture is reminiscent of that shown by certain bacteria.[4]

Summary. Electron microscope studies of the agent of feline pneumonitis suggest that the elementary bodies in nature have properties similar to those of jelly-filled sacs which in course of preparation for examination in the microscope settle to a form similar to a wrinkled pea with one flattened side. Such distorted bodies are approximately 465 mμ in diameter. They have several characteristics which resemble rickettsiae and bacteria.

15649

Pathogenizing Effect of Different Carbohydrates on *Eberthella typhosa*.

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It has long been known that the pathogenicity of bacteria for laboratory animals can be enhanced by addition of certain substances to the injected suspensions (cf. references in papers of McLeod,[1] Ercoli *et al.*[2]). Several investigators have used carbohydrates for this purpose. No systematic study of the relation between carbohydrate structure and pathogenizing potency has, however, been reported. The present communication reports experiments which form part of an investigation designed to clarify the chemical basis of pathogenizing activity in the carbohydrate group.

The experiments define the activity of selected sugars and polysaccharides as pathogenizing agents for *E. typhosa* in the mouse. Special attention is directed to the pathogenizing activity of several levans, including 2 natural sources and a preparation synthesized from sucrose by a cell-free system. The information obtained is inadequate to define finally in a positive sense the features of molecular pattern which determine pathogenizing activity in the carbohydrate group. It suffices, however, to show that several features of the chemical structure and physical character of a carbohydrate are unimportant to its rôle as a pathogenizer.

Test organism. E. typhosa (0901) served as the test microorganism. The bacteria were grown in plain broth at 37°C. the incubation lasting 24 hours.

Test of pathogenizing activity. The experiments were designed to show whether intraperitoneal injection of carbohydrate into

[1] McLeod, Ch., *Am. J. Hyg.*, Sec. B, 1941, **34**, 41 and 51.

[2] Ercoli, N., Lewis, M. N., and Harker, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 273.

a mouse has a pathogenizing effect. White mice of laboratory stock served as the experimental animal. The mice were at the time of the tests 4-5 weeks old and averaged 18 g in weight. The animals were injected intraabdominally with 0.5 cc of a suspension which consisted of a mixture of 0.05 cc of a selected dilution in broth of a 24-hour broth culture and 0.45 cc of a sterile-aqueous solution of the tested pathogenizing substance. Where no pathogenizing effect was observed, the carbohydrate concentration was increased in successive tests up to a concentration of 10% or until a concentration which gave a solution too viscous to be injected was reached. Where a pathogenizing effect was obtained the number of injected bacteria was decreased by power of 10 in successive tests until a nonlethal dose was reached. Groups of 5 mice were used at each assay level. The findings are recorded in terms of the quotient—number of animals which died within 48 hours after injection/5 (total number of mice in group). The pathogenizing activity is expressed in terms of the quotient, MLD of broth culture without added pathogenizer/MLD of broth culture in presence of added pathogenizer. In order to exclude error due to accidental death, the MLD is defined as that dose which produces a mortality rate of at least 2/5. The MLD of the broth cluture without added pathogenizer was found to be 0.4 cc. In repeats of assays of the pathogenizing potency of different substances, the maximum variation in mortality between replica tests was $\pm$ 1/5. Values of pathogenizing potency were reproducible within the limits of one logarithm integer of the broth dilution.

Materials. Sugars: pure crystalline preparations.* Galactomannan:† a preparation from carob seed. Mannan: prepared from carob galactomannan by selective hydrolysis according to the method of Wailew and Gortner.[3] Dextran: prepared in powder form from sucrose broth culture of *Leuconostoc mesenteroides* by repeated precipitation in alcohol with stirring in a Waring blendor. Levans: prepared in powder form from sucrose cultures of *Acrobacter levanicum* and *Bacillus subtilis* as described by Hestrin *et al.*[4] Cellulose I: fibril suspension prepared from cultures of *Acetobacter xylinum* as described by Aschner and Hestrin.[5] Cellulose II: acid-degraded cotton cellulose prepared by exposure of one part ether-extracted cotton wool to the action of 9 parts 60% sulfuric acid for 10 minutes at room temperature. The suspension after dilution and neutralization was dialyzed and obtained in the form of a powder by evaporation over a boiling water bath. Other materials employed: glycogen,‡ inulin,§ gum acacia,‖ agar powder,¶ mucin,** kaolin,†† rice starch, soluble starch, and pectin.‡‡

Survey of pathogenizing activity of different carbohydrates. The following carbohydrates were found to be entirely lacking in pathogenizing activity in a concentration range of 0.2-10%: pentoses—arabinose and xylose; hexoses—glucose, fructose and galactose; oligosaccharides—maltose, trehalose, lactose, cellobiose, sucrose, and raffinose; polysaccharides — galactomannan (0.2%), soluble starch (5%), rice starch (5%) and pectin (5%). The MLD in the presence of these carbohydrates except in the case of pectin was equal to that found in absence of carbohydrate. Pectin was found to be bactericidal at a concentration of 5%.

Polysaccharides which showed measurable or marked pathogenizing activity under the conditions of the test are listed in order of decreasing activity in Table I. On the basis of the maximum pathogenizing effect (pathogenizing potency) obtained 3 classes of carbohydrates can be distinguished: (a) pow-

* British Drug Houses, London.

† Kindly supplied by Dr. E. Simon, Daniel Sief Research Institute.

3 Wailew, B., and Gortner, R., *Arch. Biochem.*, 1943, **1**, 325.

4 Hestrin, S., Avineri-Shapiro, S., and Aschner, M., *Biochem. J.*, 1943, **37**, 450.

5 Aschner, M., and Hestrin, S., *Nature*, 1946, **157**, 659.

‡ Rhône-Poulenc, Paris.

§ British Drug Houses, London.

‖ Pharm. central de Belgique.

¶ May and Baker, London.

** Burroughs Wellcome, London.

†† Merck, Darmstadt.

‡‡ Commercial source unknown.

TABLE I.
Pathogenizing Activity of Polysaccharides in Presence and Absence of Kaolin.

Substances tested	Pathogenizing activity expressed as ratio: MLD without pathogenizer/MLD with pathogenizer. Concentration of carbohydrate 0.2%	0.3%	0.5%	1.0%	2.0%	5.0%	10.0%
Agar	—	8x10³	—	—	—	—	—
" with kaolin 1%	—	4x10³	—	—	—	—	—
Levan (*A. leranicum*)	—	8x10²	8x10³	8x10⁴	8x10⁵	—	—
" with kaolin 1%	—	—	—	T	T	T	T
Levan (*B. subtilis*)	—	—	—	8x10³	8x10⁴	—	—
" with kaolin 1%	—	—	—	4x10³	4x10⁴	—	—
Mucin	—	—	—	—	—	8x10⁴	8x10⁵
" with kaolin 1%	—	—	—	—	—	T	T
Dextran (*L. mesenteroides*)	—	—	—	8x10²	8x10³	—	—
" with kaolin 1%	—	—	—	—	4x10³	—	—
Cellulose I (*A. xylinum*)	—	—	—	—	8	—	—
" with kaolin 1%	—	—	—	—	4x10²	—	—
Cellulose II (Cotton)	—	—	—	—	—	80	—
" with kaolin 1%	—	—	—	—	—	4x10²	—
Gum acacia	—	—	—	—	—	1	80
" with kaolin 1%	—	—	—	—	1	4	4x10³
Mannan (carob)	—	—	—	—	—	8	—
" with kaolin 1%	—	—	—	—	—	4	—
Glycogen	—	—	—	—	1	1	8
" with kaolin 1%	—	—	—	—	40	40	4x10³
Inulin	—	—	—	—	1	1	4
" with kaolin 1%	—	—	—	—	1	40	4x10²
Dextrin	—	—	—	—	—	—	1
" with kaolin 1%	—	—	—	—	—	—	40

— = No test.
1 = MLD not lowered; 8 = MLD 8-fold lowered, etc.
T = Toxic action in absence of bacteria.

erful pathogenizers; (b) moderate pathogenizers and (c) nonpathogenizing carbohydrates. Class (a) includes 2 extracellular bacterial polysaccharides (levan and dextran), mucin and agar-agar. Members of this class showed high activity as pathogenizers also at fairly low concentration. Class (b) comprises the 2 cellulose preparations, gum acacia, glycogen, mannan and possibly should also include inulin. Class (c) comprises all the tested crystalline sugars and several of the polysaccharides (starch, galactomannan). It may be noted that the above classification of carbohydrates on the basis of pathogenizing potency is consistent with subdivision of the carbohydrates on the basis of other criteria of their pathogenizing action as described in following sections.

Copathogenizing effect of kaolin. Kaolin alone at a concentration of 1% produced no significant pathogenizing effect, the MLD being in absence of kaolin 0.4 cc and in its presence 0.2 cc. Kaolin is, however, in certain cases a powerful copathogenizer, *i.e.* it enhances the pathogenizing potency of carbohydrates which also show pathogenizing activity in its absence. The influence of kaolin on the pathogenizing potency varies consistently with the classification of carbohydrates already described. Kaolin exerts a marked effect on the pathogenizing activity of carbohydrates of class (b), but has little or no effect in class (c) and (a).

Effect of pathogenizer concentration. The effect of pathogenizer concentration has been investigated in the following: levan, mucin, gum acacia, inulin and glycogen. It is evident from the values shown in Table I that the pathogenizing activity increases at an increasing rate as the concentration of pathogenizer is increased. There is in every case a threshold level below which no activity can be found. The threshold varies markedly in the different carbohydrate classes. It is relatively low (<2%) in (a), and fairly

high (5-10%) in (b). The existence of the threshold places doubt on the general significance of negative results which may be obtained when possible pathogenizers are tested at low concentrations.

Pathogenizing activity of synthetic levan preparation. The observations on the pathogenizing activity of levan from 2 different bacterial sources suggest that levan from *A. levanicum* has significantly greater pathogenizing potency than levan from *B. subtilis*. This observation is of particular interest in view of the fact that no chemical or serological difference between different bacterial levans has hitherto been detected.[6,7] The pathogenizing action of levan prepared by the action on sucrose of a cell-free enzyme solution was therefore undertaken.

In preliminary experiments it was found that levan-free levan-sucrase solution prepared from *A. levanicum* by autolysis as described by Hestrin *et al.*[4] is toxic to mice in a dosage of 0.1 cc. Since the toxin is heat-stable and derives from a Gram-negative organism, it is probably to be regarded as endotoxin. The toxin of the enzyme preparation is precipitated by alcohol. It accompanied levan in the alcohol precipitate when levan was formed from sucrose added to the enzyme solution. Methods accordingly were sought which would permit the preparation of a nontoxic synthetic levan preparation. Two suitable methods are described below.

In one case, levan was synthesized from sucrose by incubation with levan-free levan-sucrase at 37°C for 2 days in a mixture of the following composition: 100 cc enzyme solution, 100 cc acetate buffer pH 5.0, 100 cc 15% sucrose. Toluol was added to maintain sterility. The material was precipitated with 2 parts alcohol, purified by repeated precipitation with alcohol, and dried in a vacuum desiccator. About 1.2 g of dry material was thus obtained, whereas a control mixture of enzyme and buffer without sucrose afforded 0.3 g by the same process. The yield of levan was therefore 0.9 g. To remove possibly present endotoxins the 2 products were treated according to Furth and Landsteiner[8] with hot 0.5 N NaOH for 30 minutes. The solutions were neutralized and dialyzed against tap water in cellophane bags for one day. The dialyzed solutions were precipitated with 2 volumes of alcohol. The precipitates were dried and aqueous solutions of 1 and 2% concentration were set up. Solutions of the enzyme-buffer mixture product proved to be nontoxic and nonpathogenizing, whereas the solutions of the enzyme-buffer-sucrose product, *i.e.* the synthetic levan preparation, showed no toxicity but high pathogenizing activity. The pathogenizing activity rated $8x10^2$ and $8x10^3$ at the concentration levels 1% and 2% respectively.

In a second case, advantage was taken of the fact that dilution improves the levan yield per unit of enzyme employed.[9] The reaction mixture was composed as already described except that the enzyme solution was diluted 10-fold. The yield of levan was decreased by only one-half by this 10-fold decrease of the enzyme. A levan preparation was obtained which showed no toxic effect in 2% solution even in absence of alkali pretreatment.[8] The pathogenizing activity was found to rate $8x10^2$, $8x10^2$ and $8x10^3$ at the concentration level 0.5, 1.0 and 2.0% respectively.

It may be concluded therefore that levan synthesized *in vitro* by the action of cell-free *Aerobacter* levan-sucrase on sucrose exhibits a marked pathogenizing activity independent of the presence of any alkali-labile toxic component in this preparation. The "synthetic" levan shows slightly less activity than levan prepared from a living *Aerobacter* culture.

Discussion. The experiments presented show that pathogenizing activity towards *E. typhosa* is not uncommon in polysaccharides. Of a total of 15 polysaccharides investigated, high pathogenizing potency was found in 5, moderate activity in 6, and little or no activity in the remainder. On the other hand,

6 Genghof, D. S., Hehre, E. J., and Neill, J. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 339.

7 Hehre, E. J., Genghof, S. D., and Neill, J. M., *J. Immunol.*, 1945, **51**, 5.

8 Furth, J., and Landsteiner, K., *J. Exp. Med.*, 1928, **47**, 171.

9 Avineri-Shapiro, S., and Hestrin, S., *Biochem. J.*, 1945, **39**, 167.

not one member of a group of 10 oligosaccharides and simpler sugars examined showed pathogenizing activity. It is appropriate in the light of these findings to inquire what physical and chemical properties in a carbohydrate determine its activity.

The hypothesis has been presented that viscosity is an overriding determinant of the pathogenizing activity of an injected substance. Experiments by several investigators have tended, however, to throw doubt on this conclusion. The experiments here reported confirm this criticism. Among the carbohydrates examined by us, no consistent relationship between viscosity and pathogenizing activity can be observed. In the polysaccharide group with marked pathogenizing activity, dextran with a viscosity at a concentration of 2% about 10 times that of levan shows far less pathogenizing activity than does levan at the same concentration. Again, in the group of polysaccharides with low pathogenizing activity, a pathogenizing potency which is roughly constant is associated with widely fluctuating viscosities. Finally, it may be noted that the inactive polysaccharide group includes several substances of very high viscosity (galactomannan, mannan, starch). It is clear, therefore, that viscosity is not an overriding determinant of pathogenizing activity and that it does not *per se* confer pathogenizing activity on an injected substance.

Since a pathogenizing activity which is absent in simple sugars and oligosaccharides can be present in their polymers, it is suggested that pathogenizing activity is a function of patterns which are specific to the colloidal state. It is significant in this respect that different polysaccharides with a common hexose unit and similar features of chemical structure can present widely different pathogenizing activity (levan from *A. levanicum* vs. levan from *B. subtilis* vs. inulin: starch vs. dextran, etc.)

Pathogenizing activity seems to be dependent only in a minor degree on the structure, configuration and manner of linkage of the individual repeating unit or on the presence of polar groups in a carbohydrate polymer. Thus it has been found that either high or moderate pathogenizing activity may be manifested by simple polysaccharides of such groups as glucan (dextran, cellulose preparations, glycogen), fructan (levans, inulin), and mannan (carob preparation) and by complex polysaccharides with polar groupings (agar, gum acacia, mucin). Our pathogenizing carbohydrates include both α and β linkage types, furanosidic as well as pyranosidic units, and intersaccharidic C-C linkages of the 1-4 (cellulose, glycogen), 1-6 (dextran), 2-1 (inulin) as well as 2-6 (levan) types.

In an investigation of the pathogenizing potency of different fractions of mucin, Anderson and Oag[10] found that whereas marked activity is shown by protein fractions no pathogenizing activity is presented by the purified carbohydrate fraction of mucin. This result suggests that the pathogenizing activity of certain carbohydrates may be due to the presence of foreign substances in these preparations. In view of the fact that pathogenizing activity is manifested by polysaccharides from widely different sources, this possibility must be considered as most unlikely. In the case of levan it has been possible to exclude it. The demonstration of pathogenizing activity in a levan obtained in defined reaction conditions by the action of cell-free enzyme on pure sucrose affords proof that pathogenizing activity is an intrinsic property of levan.

Summary. The pathogenizing action of 15 polysaccharides and of 11 simple sugars and oligosaccharides on *Eberthella typhosa* in the mouse has been described. High pathogenizing activity was shown by the following carbohydrates: levan, dextran, mucin, and agar-agar. Moderate activity was shown by the following: cellulose preparations, gum acacia, glycogen and mannan. Inclusion of kaolin in the injected suspension enhanced the pathogenizing action of this group. Levans from 2 bacterial sources *Aerobacter levanicum* and *Bacillus subtilis* showed different degrees of pathogenizing activity. Levan synthesized *in vitro* by the

[10] Anderson, C. G., and Oag, R. K., *Brit. J. Exp. Path.*, 1939, **20**, 25.

action of cell-free *Aerobacter* enzyme showed marked pathogenizing activity. The pathogenizing activity must be, therefore, an intrinsic property of this levan.

It is suggested that the pathogenizing activity of a polysaccharide is a function of patterns which are specific to the colloidal state. The activity depends only in minor degree on the structure, configuration and manner of linkage of the individual repeating unit or on the presence of polar groupings in the carbohydrate polymer. There is no consistent relationship between the viscosity of the injected carbohydrate solution and the pathogenizing activity which it manifests.

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Electroshock Therapy by Stimulation of Discrete Cortical Sites with Small Electrodes.*

ROBERT G. HEATH† AND EDWARD C. NORMAN. (Introduced by Fred A. Mettler.)

From Pennsylvania Hospital for Nervous and Mental Diseases, Philadelphia, Pa.

Among the many phenomena observed during and following electroshock therapy are convulsion, loss of memory and profound stimulation of the autonomic nervous system. By using small electrodes (8 mm in diameter as contrasted to conventional electrodes of 5 cm^2) it was found that no convulsion resulted when the stimulus was applied through regions some distance from the motor strip, although the current used was sufficient to produce convulsions when applied to the motor strip itself. The conventional shock machine was used in the experiment. Fairly accurate control of current was possible by measuring the resistance of the subject.

Some degree of localization of the stimulation was possible. Observations made following stimulation of several chosen sites indicated that stimuli over the various regions produced different results. Correlating these observations with clinical results and information concerning other organic treatments used in mental disorders, has made possible some speculation concerning the factors which make electroshock treatment effective. It has also offered directions for further investigations into the mechanism of the psychoses.

Sites for stimulation through the frontal area were chosen in accordance to external skull landmarks. Site A was high on Area 4 (Brodmann) just posterior to the interauricular line. Site B was roughly through Brodmann's Area 11. Site C was roughly through Brodmann's Areas 9 and 10. Site D was a point midway between the orbit and auditory canal and 1 cm above the upper border of the zygomatic process. (Brodmann Area 38).

Observations of results following stimulation of the selected sites will be presented according to:

(A) Motor phenomena
(B) Autonomic nervous system phenomena
(C) Disturbance of intellectual function
(D) Clinical results

(A) Motor phenomena. Stimulation of Site A or Area 4 of Brodmann resulted in a typical tonic and clonic convulsion lasting 40 seconds to one minute. Stimulation of the other sites produced with considerable regularity a single extension "jerk" of all extremities.

(B) Autonomic nervous system phenom-

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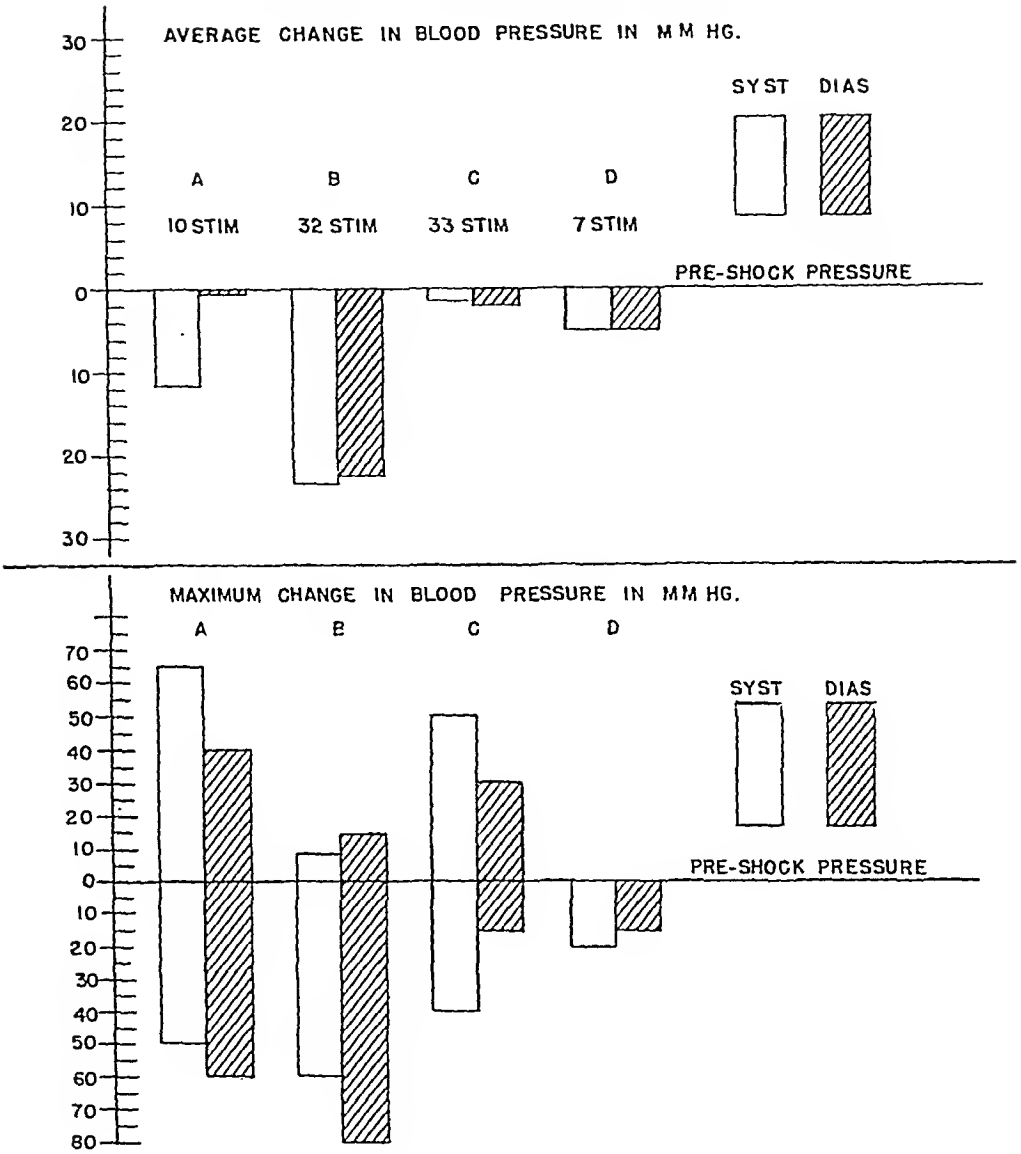

FIG. 1.
A. Average change in blood pressure. B. Maximum change in blood pressure.

ena. 1. Blood pressure (Fig. 1A and 1B).

Site A—Ten stimulations with convulsion. There was a rise of systolic and diastolic pressure in 2 cases and a fall in 8. Average systolic change was —11.5 mm; extremes +65 mm and —50 mm. Average diastolic change —0.5 mm; extremes +40 mm and —60 mm.

The time at which blood pressure was recorded in relation to the first deep respiration probably accounts for the inconsistent finding of 2 rises and 8 falls in pressure. With the cessation of respiration accompanying the tonic and clonic movements, there is a rise of pressure. With the first deep breath following the convulsion there is a rapid fall.

Site B—32 stimulations; blood pressure increased in 2, dropped in 30; average systolic change —23.6 mm; extremes —60

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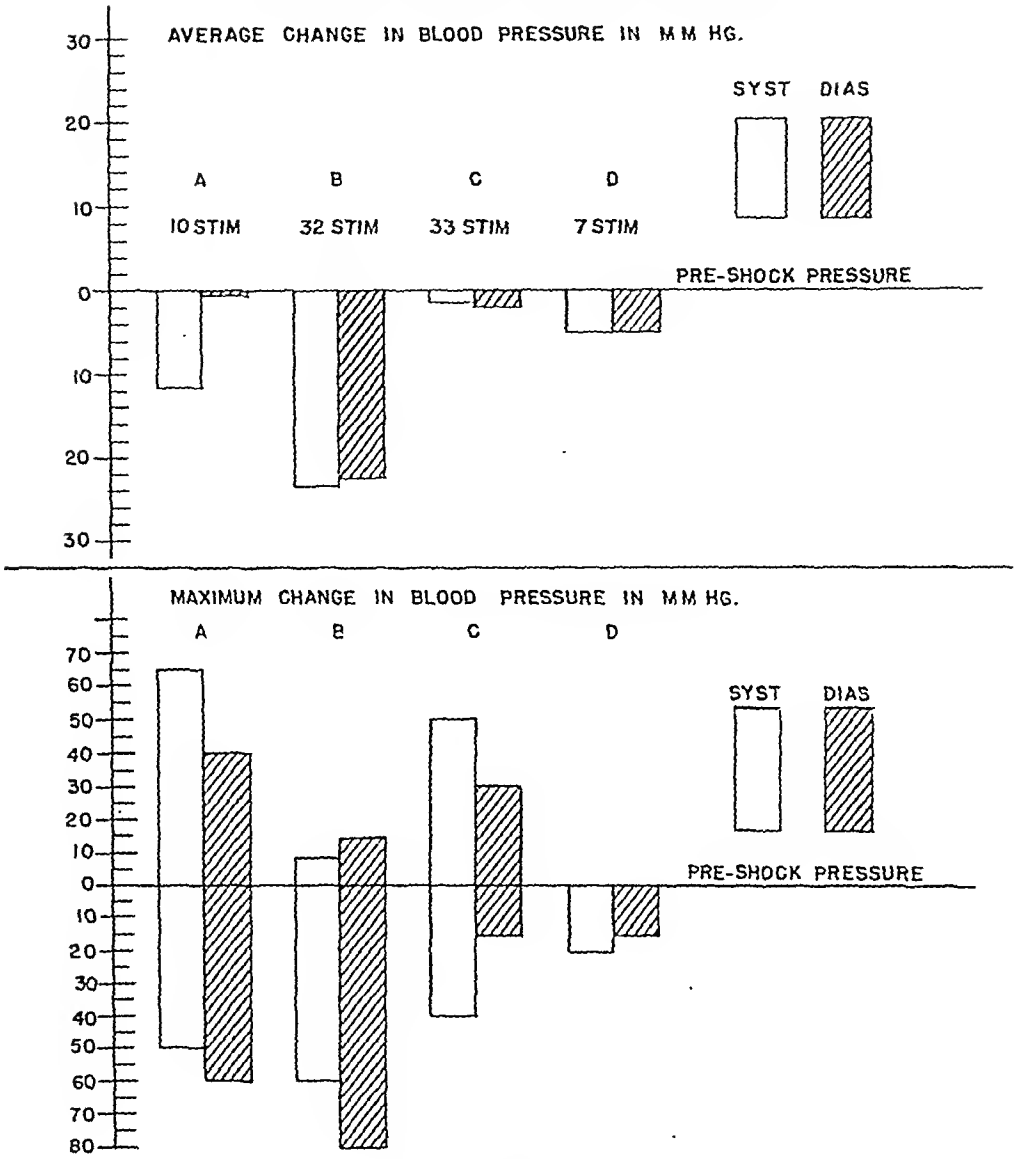

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Site B—32 stimulations; blood pressure increased in 2, dropped in 30; average systolic change —23.6 mm; extremes —60

mm and +8 mm. Average diastolic change —22.3 mm; extremes —80 mm and +14 mm.

Site C—33 stimulations; average systolic change —1.4 mm; extremes +50 mm and —40 mm. Average diastolic change —2.0 mm; extremes +30 mm and —15 mm.

Six patients were stimulated in both Site B and Site C. In 3 of these cases Site B stimulation produced a fall, whereas C caused a rise. In the other 3 patients a drop was recorded after stimulation over both Site B and Site C. The fall after stimulation of Site B was, however, considerably greater.

Site D—7 stimulations. On 5 occasions there was no change. Twice the pressure dropped. Average change —5 mm in both systolic and diastolic pressure; extremes —20 mm systolic and —15 mm diastolic.

Summary. Stimulation at Site B produced a marked depressor effect after 30 of 32 stimuli. The effect of stimulation at Site C was not so consistent, the pressor response being noted with about the same frequency as the depressor. Over Site D there was no change in pressure following 70% of the stimulations. The effect of motor response on blood pressure changes is not important here as it was the same with excitation of each of the 3 sites.

2. Pulse rate. Recordings of pulse following stimulation of Site A are of no value because of the accompanying convulsion. After stimulation of Site B in 10 patients the rate was slowed in 7 and increased in 3. In contrast, after stimulation at Site C the rate speeded in 7 and slowed in 3. The rate was consistently slowed in 5 cases stimulated over Site D.

3. Pupillary response. Observations were made on 5 patients after stimulation at Site A and, on every occasion, the pupils were observed to dilate. Following stimulation at Site B, contraction was noted in 26 cases and dilation in but 3. Stimulation at Site C produced contraction in 22 cases and dilation in 23. Of 20 cases stimulated over Site D, 15 showed contraction and 5 dilation.

4. Peripheral vasomotor reaction. During the convulsion following Site A stimulation, the vasomotor reactions were not consistent. Usually, however, there was pallor immediately after stimulation, followed in a few seconds by a generalized flush. Toward the end of the convulsion cyanosis set in; but was followed again by flush with the initial deep respiration. One hundred ten stimulations over Site B were followed in every case by marked flushing of the skin. Almost invariably lacrimation was also noted. In 50 observations after stimulation at Site C, the flush was noted in two-thirds, but in the other one-third (15 cases) there was pallor of the skin. In 5 of the 15 cases which showed pallor, there was also profuse sweating. Site D stimulation produced very little change in peripheral vasomotor status.

5. Respiration. Slowing of respiratory rate, and often a period of prolonged apnea with mild cyanosis, was noted consistently following Site D stimulation. As Area 6B (Brodmann) was approached, this became more pronounced. Bucy[1] stimulated this area during a craniotomy on a patient and found that respiration temporarily ceased. His paper also refers to other work on cortical respiratory centers. Our findings support his conclusion that this area is a respiratory inhibitory center. It must, however, be considered that the apnea may have resulted from spread to medullary centers.

The only conclusion that can be drawn from these observations of autonomic phenomena occurring with focal stimulation of the brain through the skull is that there is a cortical autonomic level which when stimulated does exert a strong effect. The finding of different responses to stimulation of the various sites suggest localized areas of autonomic representation. However, by such diffuse stimulation one cannot hope to accurately localize autonomic function.

(C) Disturbance of intellectual function. Immediate effects: Following conventional electroshock treatment the patient remains dazed for 15 to 20 minutes. He has a blank, far away stare in his eyes and does not respond to the usual stimuli. He is often restless, and thrashes about aimlessly in bed. At times there is rather violent

[1] Bucy, P. C., and Chase, T. J., *J. Nervous and Mental Diseases*, 1936, **84**, 156.

motor activity. This marked confusion usually clears in 30 minutes to a few hours.

A review of the literature indicates that about 80% of cases develop a more lasting memory impairment which remains a few weeks to several months. Several authors[2,3] have suggested that some intellectual impairment may be irreversible. A few cases[3] are cited in which disturbances of memory remain 2 to 3 years following treatment.

Following stimulation over Site A (motor strip with convulsion) the recovery was more rapid. Patients could answer simple questions in less than 5 minutes. They did not develop the same dazed, far away look nor the marked hyperkinesia.

After stimulation of Sites B and C patients complained of anxiety and discomfort and were reluctant to continue treatment. There was no evident intellectual impairment.

Stimulation at Site D produced amnesia for several minutes. The patients appeared dazed and confused like those who had received conventional shock. The effect was, however, of shorter duration.

(D) Clinical results. Site A—(motor strip)—Results from stimulation of this area were essentially the same as with conventional therapy (*i.e.*, it was very effective in the treatment of affective psychoses—involutionals and manic depressive - depressed); with equivocal results in manic depressive—manic and schizophrenia. This method of administering convulsion did, we believe, possess an added advantage. Inasmuch as there was little memory loss and confusion, it was possible to administer psychotherapy in conjunction with electroshock therapy.

A case history will be briefly outlined to illustrate this: A 27-year-old, white female with a history of a mild depression 2 years previously, from which she spontaneously recovered in the course of 6 months, witnessed the bombing of Pearl Harbor during which her fiance was killed. After repatriation to the U. S. she spent 2 months nursing her fiance's mother through a nervous breakdown. She then took a job as stenographer at the San Diego Naval Base so as to be close to his friends. In an effort to forget she began to drink heavily and her morals became lax. (She had been raised strictly in the Catholic faith and attended Catholic boarding schools). Eventually pregnancy resulted and, taking the advice of a friend, she had an abortion performed. It was while convalescing from this that her psychosis developed, taking the form of schizo-depression with auditory hallucinations, delusions of persecution, self-abasement and attempted suicide. She entered the Pennsylvania Hospital a few days later and after the preliminary workup, electroshock treatments were begun. After 3 or 4 treatments she was so confused as to be unable to give her own name. Although all symptoms of psychosis disappeared several additional treatments were given. Approximately 7 to 10 days after the last treatment the confusion began to subside but, as her memory returned and before it was possible to begin psychotherapy, the symptoms of the psychosis returned. This procedure was repeated twice more, *i.e.*, treatment with improvement while confused, followed by relapse as memory returned. After the third relapse, shock treatment was instituted with small electrodes over the motor strip. No confusion or memory loss resulted and it was possible to administer psychotherapy shortly after the conclusion of the convulsion. The patient talked out her problems and remained well.

Site B stimulation was considerably less effective than was stimulation which produced a generalized convulsion as with Site A. Some patients, however, were benefited clinically and one made a complete recovery, but not until 20 to 25 treatments or many more than the customary number were given.

Site C—Results of stimulation of this area were essentially the same as those obtained from stimulation of Site B.

Site D—Stimulation of this area alone produced no clinical improvement. Analysis of data thus far accumulated suggests that:

1. Amnesia is not necessary to produce clinical improvement.

2. Respiratory complications can be les-

[2] Levy, N. A., Serota, H. M., and Grinker, R. R., *Arch. Neurol. and Psych.*, 1942, **47**, 1009.

[3] Brody, M. B., *J. Men. Sci.*, 1944, **90**, 777.

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TABLE I.
Summary of Clinical Results in Electro-shock Treatment with Small Electrodes.

	Manic Depressive-Depressed					Involutional Melancholia				
Site	A	B	C	D	Multiple stimuli	A	B	C	D	Multiple stimuli
Well	8 63%	1 12.5%	0	0	0	5 83%	1 16%	1 16%	0	2 50%
Improved	2 27%	4 50 %	3 50%	0	0	1 17%	2 34%	2 34%	0	1 25%
No change	1 10%	3 37.5%	3 50%	2 100%	1 100%	0	3 50%	3 50%	2 100%	1 25%
Total No. of cases treated	11	8	6	2	1	6	6	6	2	4

a private institution and felt any therapy was preferable to state hospitalization.

Treatment was begun in May, 1943. She received 7 stimuli on each of 34 consecutive days, exclusive of Sunday, the first being directed through Site D so as to produce amnesia for the treatment. At the end of these periods all symptoms of her illness subsided and shortly she was discharged to her daughter. Regular follow-up letters indicate she continues to remain well.

Analysis of results of multiple stimuli. Although our cases are too few in number to permit definite conclusions the evidence is sufficient to make the following statements:

1. With multiple stimuli to that part of the frontal cortex that has autonomic representation the results appear to be better than with single stimulation.

2. The increased percentage of cures tends to support our hypothesis that stimulation of the autonomic nervous system may play a large role in the clinical results in electroshock therapy.

Other evidence to indicate the role of the autonomic nervous system in mental disorders.

1. The suggestion emphasized by these procedures, that the autonomic nervous system plays a major role in the affective mental disorders, is supported by frequent reports of findings of altered autonomic function in these psychoses. For example, in the involutional case there is insomnia, agitation, often elevated blood pressure, inability to eat, etc. Altered autonomic functions in depressed cases are also well known.

2. Clinical results reported with electronarcosis are at least as favorable as those reported with electroshock in the treatment of the affective disorders and are considerably better in the treatment of schizophrenia. In electronarcosis a smaller current than that used in electroshock is administered over a prolonged period. (60-cycle a.c. machine—initial current of 150 to 250 milliamperes for 30 seconds then decrease to level which permits respiration *i.e.*, 45 to 90 m.a. for up to 20 minutes). Frostig *et al.*[6] describe autonomic effects, motor phenomena and loss of consciousness with this procedure. The motor effects are perhaps less pronounced than with electroshock. There is an initial extensor spasm, then a few tonic and clonic movements, after which all variation between narcosis and marked hyperkinesia are seen for the duration of the treatment. The autonomic effects are quite variable. They are much more prolonged than with electroshock (dependent upon duration of treatment). The electrodes used in this treatment are 5 cm x 5 cm and the placement is over the temporal region. An extensive area of the brain is, therefore, stimulated much as in conventional electroshock.

3. Lobotomy, another effective form of treatment of the psychoses is directed toward affecting the autonomic nervous system. By this procedure pathways are cut which link the prefrontal cortex with the thalamus and lower centers, thereby isolating the autonomic cortex. Watts and Freeman[7] in describing

[6] Frostig, J. P., Van Harreveld, A., Reznick, S., Tyler, D. B., and Wiersma, C. A. G., *Arch. Neurol. and Psych.*, 1944, **51**, 232.

[7] Freeman, W., and Watts, J. W., *Tr. N. Y. Acad. Sci.*, 1944, **6**, 284.

sened by avoiding Area 6B (Brodmann), the cortical respiratory inhibitory center.

3. Some benefits are derived from stimulation through the frontal lobes without convulsion, although results are considerably below those achieved with convulsion.

4. Stimulation through Site D (temporal cortex, basal ganglia and hypothalamus) which produced memory loss but little effect on the autonomic nervous system (except pupillary responses) resulted in no clinical improvement.

Frontal lobe stimulation (at Sites B and C) produced some clinical improvement. It was, however, considerably less effective than motor strip (Site A) stimulation. The autonomic nervous system was affected in stimulation of all these sites. The autonomic cortex is reported to extend from the central sulcus anteriorly to the frontal pole.[4,5] There is a marked difference in the duration of the effects of the stimulus in these 2 regions. When Sites B and C were stimulated the effect was for the duration of the stimulus only (0.3 to 0.6 second). When Site A was stimulated producing a convulsion there was a spread of the excitation over the entire motor strip and it continued throughout the convulsion (45 sec. to 1 min.)

It seemed possible, therefore, that profound excitation of the autonomic nervous system might be the reason for clinical improvement. If this were the case, it might be possible to obtain autonomic stimulation and, therefore, clinical improvement without convulsion by repeated stimulation through the frontal lobe anterior to the motor strip.

In an effort to further investigate this assumption repeated stimuli were given through the frontal lobes anterior to the motor strip. In this way more prolonged autonomic stimulation could be obtained, still without convulsion, and if the forementioned speculations were correct, that clinical results were obtained from excitation of the autonomic nervous system, our percentage of cures should have increased.

Six stimuli about 10 seconds apart were administered throughout the frontal area. The first patient resented this treatment and refused to permit it because of the intense anxiety produced by each shock (as mentioned above in discussion of results of Site B and C stimulations). To overcome this complication we first stimulated Site D. Amnesia resulted and, thereafter, the patient had no recollection of the treatment and submitted uncomplainingly (much as in conventional therapy) to further stimulation at other sites.

Before results are discussed it should be pointed out that even with 6 shocks of 0.5 second each, the duration of autonomic excitation is considerably less than that seen in the generalized convulsion (45 sec. to 1 min.)

Table I gives a comparison of the clinical results obtained with stimulation of the various sites. The one manic depressive-depressed case treated unsuccessfully with multiple stimuli was subsequently given a full course of conventional shock treatment with no improvement. He was transferred to a state institution.

Case history to illustrate this procedure. A 69-year-old, white female. Prepsychotic personality; meticulous, rigid. Married but not happy; 2 children. Because of financial difficulties after husband's death, lived with sister for 5 years before onset of illness. Did not get along with sister.

Present illness. Ostensibly precipitated by a flood which damaged her home in January 1943; became extremely agitated and depressed; threatened suicide; self-accusatory; believed herself destitute and penniless; entered Pennsylvania Hospital March 1943.

Physical examination. 1. Old fracture of right hip with shortening of right leg.

2. Hypertrophic arthritis.

X-ray of spine: Degenerative changes and narrowing of discs.

X-ray of hip: Old osteochondritis with obliteration of the joint space.

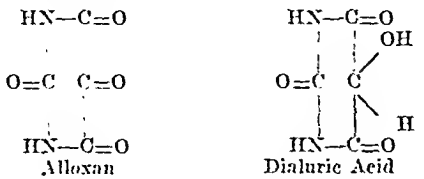
Orthodox shock therapy was advised against by the roentgenologist. The family could ill afford prolonged hospitalization in

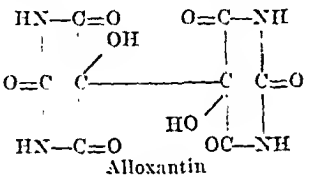
[4] Kennard, M. A., *Illinois Monographs in the Medical Sciences*, 1944, 4, 295.

[5] Fulton, J. F., *Physiology of the Nervous System*, second edition, New York, Oxford University Press.

Determinations of blood sugar were made routinely by the micro procedure of Folin and Malmros[7] and in one instance (Rabbit No. 1) checked by a micro copper method referred to in an earlier paper.[8]

The chemical relationship between alloxan, dialuric acid and alloxantin is shown in the formulas below:





Dialuric acid is a reduction product of alloxan and may be oxidized to alloxan. That oxidation of dialuric acid to alloxan occurs easily in *very dilute solutions* in the presence of air has been emphasized by Archibald.[9] However, by applying Archibald's differentiating phosphotungstic acid test[9] to a warm 1.5% solution of dialuric acid (the concentration used in the experimental work) we have found less than 10% oxidation to alloxan occurring during the first 15 minutes at room temperature and only about 20% during a half hour at room temperature and reheating.

These results indicate that a solution of dialuric acid prepared as indicated contains during the first 30 minutes after its preparation at least 80% dialuric acid and probably no more than 10-20% alloxan.

Results. Chemical. The blood sugar results of the animals injected with 250 or 200 mg/kg are shown in Fig. 1. Three of these rabbits developed severe diabetes, one (No. 3) developed an initial blood sugar curve similar to the alloxan-injected animal but died 12 hours after the injection. Rabbit No. 5, though showing an initial hyperglycemia, did not develop diabetes.

Rabbits No. 6, No. 7 and No. 8, injected with 150 mg/kg, did not develop diabetes. No. 8 showed a tendency toward the transitory type of diabetes with one blood sugar of 278 mg % on the third day following injection, but a glucose tolerance on this rabbit 40 days after the injection was normal.

These results suggest that the rabbit requires a slightly larger dose of dialuric acid to produce permanent diabetes than of alloxan. If dialuric acid is given in 200-250 mg/kg doses, however, the blood sugar changes produced seem to parallel those observed in alloxan-injected animals.

Histology. Pancreas. The early stages of the pancreatic lesion were not studied since our purpose was to compare the fully developed lesions due to dialuric acid and to alloxan. The pancreas of the rabbits rendered diabetic by the injection of dialuric acid was characterized by paucity of islets and the small size of those remaining. A large percentage of the islets had disappeared completely. Their former sites were identified only with difficulty, being marked only by small masses of connective tissue without inflammatory cellular infiltration or residual traces of islet cells. In those islets which remained, the beta cells had entirely disappeared without concomitant infiltration with lymphocytes or polymorphonuclear leukocytes. Occasional alpha cells of normal morphology were seen, but more numerous alpha cells had pyknotic nuclei and agranular cytoplasm at the stage studied. The acinar and ductile tissues, stroma and blood vessels of the pancreas were within normal limits. The lesion was thus extremely similar to that seen in the pancreas in alloxan diabetes,[10] the only distinction being that some degenerating islet cells were present even when the rabbits were well established in diabetes.

Kidneys. There was considerable damage

[7] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

[8] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, 1945, **157**, 525.

[9] Archibald, R. M., *J. Biol. Chem.*, 1945, **158**, 347.

[10] Bailey, O. T., Bailey, C. C., and Hagen, W. H., *Am. J. Med. Sci.*, 1944, **208**, 450.

the operation state that immediately there is a disappearance of nervous tension. Although memory is intact and conflicts still exist, they cause little concern. They report their patients are able to formulate ideas but lack the accompanying emotional drive that is necessary to put them into effect. As a result their productivity is usually markedly impaired.

Summary. By using small electrodes in electroshock treatment it was possible to localize the stimulation sufficiently to separate some of the many phenomena that occur with conventional electroshock therapy. The small amount of data thus far accumulated seems to indicate: (1) Memory loss and convulsion are probably not necessary to produce clinical improvement. (2) Stimulation of cortical autonomic centers seems to be the most important factor in producing clinical improvement in the affective disorders with electroshock.

15651

Diabetes Mellitus in Rabbits Injected with Dialuric Acid.*

C. CABELL BAILEY, ORVILLE T. BAILEY, AND RACHEL S. LEECH.
(Introduced by Elliott P. Joslin.)

From the George F. Baker Clinic, New England Deaconess Hospital, and the Department of Pathology, Harvard Medical School.

Since the discovery that alloxan produces diabetes when injected into animals[1-3] other chemically related substances have been studied for possible diabetogenic action. Until recently no chemical with such action has been found.

Koref, Vargos, Rodriguez and Telchi[4] reported the production of diabetes with alloxantin and confirmation of this work has already been published from this laboratory.[5] Recently, Bruckman and Wertheimer[6] have found that besides alloxantin methylalloxan, dialuric acid, methyl dialuric acid and dimethyl alloxantin produce diabetes when injected into rats. The present investigation reports the production of diabetes in the rabbit with dialuric acid and compares the diabetes so produced, clinically and histologically, with the diabetes produced with alloxan.

Experimental. Eight chinchilla male rabbits each weighing 1500-2000 g were injected intravenously with dialuric acid in doses of 150, 200 or 250 mg per kg. The rabbits were not fasted at any time during the experimental period and in all but one instance the injection was made with the animal under nembutal anesthesia. Anesthesia seemed advisable since the dialuric acid was injected as a hot solution, owing to its insolubility at lower temperatures.

The solution of dialuric acid was prepared as a 1.5% solution in nearly boiling distilled water. The solution was cooled to a temperature of 40°-50° C and injected immediately. It was found necessary to keep the solution at this temperature to avoid recrystallization of the dialuric acid which tended to occur at room temperature.

Blood samples were taken from the ear veins before and at frequent intervals following the injection of the dialuric acid.

* This investigation has been aided by a grant from the American Cyanamid Company, who also supplied the dialuric acid.

1 Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

2 Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

3 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

4 Koref, O., Vargos, L., Rodriquez, F. H., and Telchi, A., *Endocrinology*, 1944, **35**, 391.

5 Bailey, C. C., Bailey, O. T., and Leech, R. S., *Bull. New Eng. Med. Center*, 1945, **7**, 59.

6 Bruckmann, G., and Wertheimer, E., *Nature*, 1945, **155**, 267.

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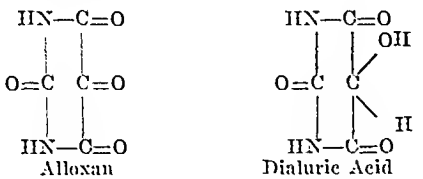

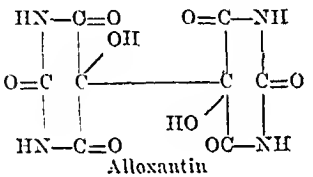

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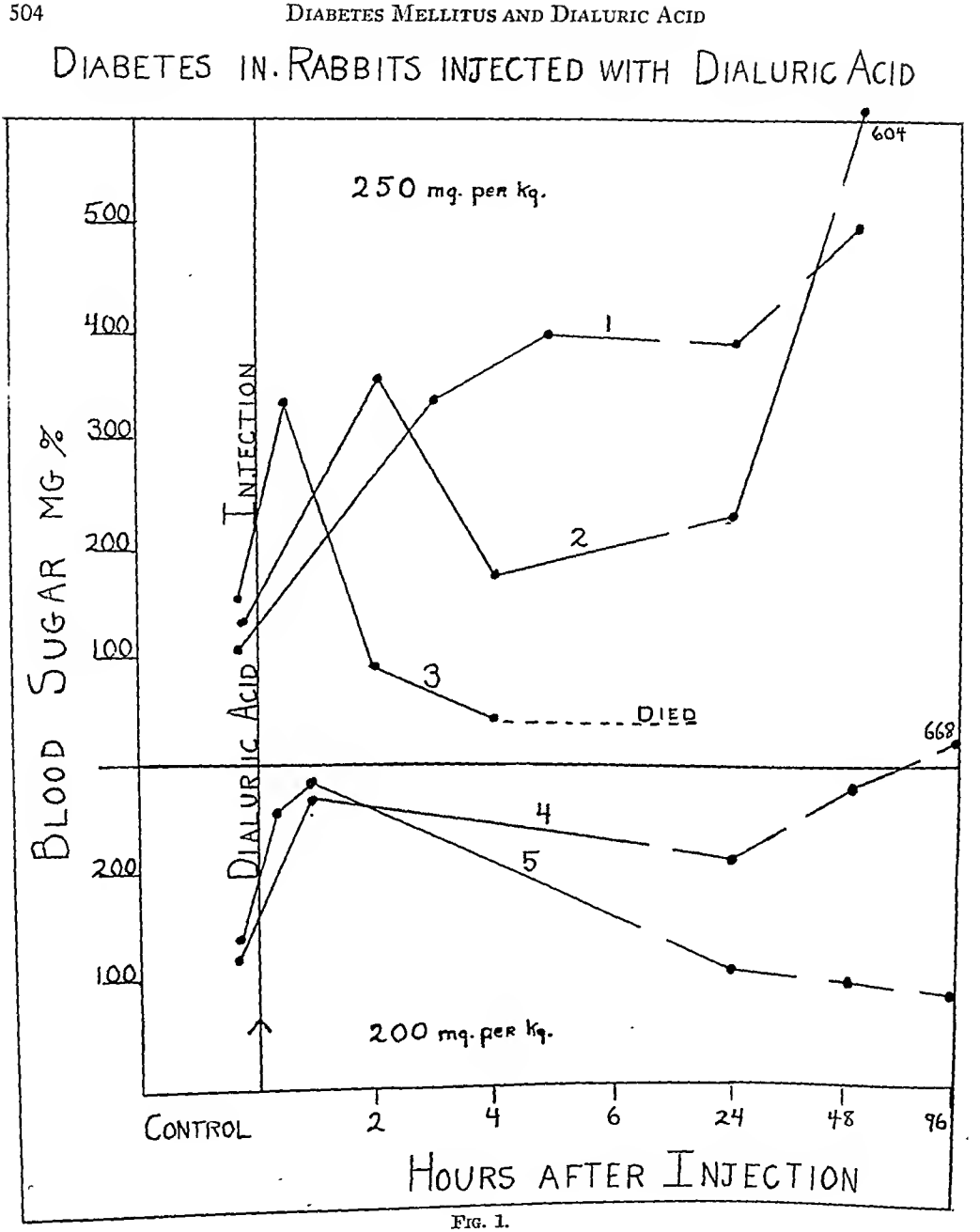

FIG. 1.

to the renal convoluted tubules. The tubular epithelia cells were swollen and the nuclei of many of them were pyknotic. They contained protein precipitate. The glomeruli, convoluted tubules, pelvic epithelium and blood vessels were within normal limits. The character of the renal damage was similar to that seen in alloxan diabetes. Since the extent of the kidney changes varied considerably in our large series of rabbits with alloxan diabetes, it is difficult to compare the degree of renal injury resulting from the injection of the 2 substances. In the material at hand, the renal changes due to

dialuric acid seemed to be at least as great and possibly greater than those produced by alloxan.

Other Organs. The liver was somewhat congested, but there was no fatty infiltration. Other organs contained no lesions not found in control animals.

The adrenal showed no infiltration with polymorphonuclear leukocytes or other pathologic changes.

In Rabbit No. 3, which developed severe hypoglycemia and died during the night after injection of dialuric acid, the lesions were at an earlier stage than those of the diabetic animals. In many islets the central beta cells were pyknotic and the cytoplasm agranular, while some alpha cells at the periphery were essentially normal. In other islets all the cells had pyknotic nuclei. Again the degeneration and disintegration of the islet cells were unaccompanied by infiltration of inflammatory cells. The histologic features of other organs were similar to those already described in the diabetic animals.

The rabbit which developed transitory diabetes (No. 8) showed much less marked changes in the pancreatic islets. The alpha cells and many of the beta cells were normal in appearance. Some of the beta cells were pyknotic, while others were judged to have disappeared, since they were less numerous than in control rabbits. There was no inflammatory cellular infiltration. No hydroptic degeneration or mitoses were seen in the islet cells. The epithelial cells of the convoluted tubules of the kidney were swollen but there was less pyknosis and less protein precipitate in the lumen than in the diabetic animals.

In the rabbits (No. 5, 6, 7) not developing diabetes, either permanent or transitory, there were only a few pyknotic beta cells in some of the pancreatic islets and the other pancreatic changes were absent. The degree of renal damage, however, was as great as in the rabbits developing transitory diabetes.

These studies have been based on tissues fixed in acetic Zenker's solution and stained with phloxine-methylene blue and with Gomori's method for the cells of the pancreatic islets.

Discussion. Clinically the diabetes produced in rabbits by dialuric acid and by alloxan was identical except for the fact that a slightly larger dose is necessary when employing dialuric acid.

The triphasic blood sugar curve consisting of an initial transitory hyperglycemia followed by hypoglycemia and finally a permanent hyperglycemia or diabetes is found after the injection of either dialuric or alloxan. Permanent diabetes may be produced with either drug and with the use of smaller doses transitory diabetes may occur.

The histologic changes described as the result of injection of dialuric acid are closely similar to those due to the injection of alloxan. The few variations mentioned are trifling when considered from the functional point of view. The cardinal features of alloxan diabetes in the rabbit—necrosis of islet cells with survival of a few alpha cells, moderate to mild injury to the renal convoluted tubules, with only minor changes in other organs—are duplicated in dialuric acid diabetes. Such evidence as can be gleaned from these experiments suggests that dialuric acid diabetes, like alloxan diabetes, is due to a lesion of the islets of Langerhans, which is degenerative from its inception.

Since dialuric acid may be obtained as a reduction product of alloxan, it might be questioned whether one of these substances is converted into the other in the body of the experimental animal before the lesions are initiated. The present series of experiments throws no light on this question other than to indicate that the lesions caused by the 2 substances are similar and are consistent with either view.

Dialuric acid is not recommended as a substitute for alloxan as an experimental technic for the production of diabetes, because it must be injected in a warm solution owing to its low solubility. Except for the extra labor involved, however, dialuric acid and alloxan may be regarded as interchangeable as diabetogenic agents.

Summary. Dialuric acid when injected intravenously into rabbits produces diabetes which is indistinguishable clinically or pathologically from alloxan diabetes.

15652

Action of Urea and Some of Its Derivatives on Bacteria. V. Antibacterial Activity of Methyl- and Thiourea.*

LOUIS WEINSTEIN. (Introduced by C. S. Keefer.)

From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine, Boston.

It has been reported[1-3] that urea and urethane are highly bacteriostatic and bactericidal for many Gram-negative and several Gram-positive organisms, potentiate moderately the activity of sulfonamides, inhibit *p*-aminobenzoic acid moderately, and increase the solubility of sulfanilamide and sulfathiazole. To determine whether increased bacteriostatic potency results from substitution of one of the NH_2 groups in urea by $-CH_3$ radicals, as was suggested by the superiority of urethane over urea, other derivatives of carbamide were investigated. The present paper deals with a study of antibacterial and anti-*p*-aminobenzoic acid activity of methyl and thio derivatives of urea, the $-CH_3$ group replacing one $-NH_2$ in the first compound and —S being substituted for the =O in urea in the second.

A review of the literature reveals no information concerning the activity of methyl urea against microorganisms. Thiourea is reported[4] to be antibacterial and to increase the effectiveness of sulfathiazole. This action was thought to be synergistic.

Methods. The methods used in the studies described in this paper are similar to those detailed in the previous ones of this series and, therefore, will not be described here. Veal-infusion broth with added horse serum is designated as VIB-S. The pH of 5% methyl- and thiourea solutions in VIB-S was found to be 7.2 to 7.25.

Results. I. *The Bacteriostatic Effects of Methyl Urea.* A study of the bacteriostatic effect of methyl urea in amounts varying from 2 to 8% in VIB-S revealed that a concentration of approximately 6% produced suppression of growth of several Gram-negative organisms. *Ps. aeruginosa* was completely inhibited by a 4% solution of the drug. Moderate bacteriostasis of *E. cali, S. schottmulleri, P. vulgaris,* and *E. typhi* resulted from exposure to 5% methyl urea. *P. vulgaris* was partially inhibited by 6% and an 8% solution completely arrested growth for 96 hours.

In a synthetic medium,[5] the concentration

TABLE I.

Effect of Varying Concentrations of Methyl Urea on Growth of Various Organisms in Different Media.

			Concentration of methyl carbamate — Growth at various periods									
			6%		5%		4%		3%		2%	No drug
Medium	Organisms inoculated	hrs	48	96	48	96	48	96	48	96	48	96	48	96	48	96
VIB-S	*E. coli*—6500		0	0	0	3+	2+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	S. schottmulleri—1050		0	0	0	2+	1+	3+	4+	4+	4+	4+	4+	4+	4+	4+
	Ps. aeruginosa—29,000		0	0	0	0	0	0	1+	3+	4+	4+	4+	4+	4+	4+
	*P. vulgaris**—34,000		0	2+	1+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	E. typhi—525		0	0	0	0	0	0	1+	3+	3+	4+	4+	4+	4+	4+
Synthetic medium	*E. coli*—22,000,000		0	0	0	0	0	0	0	3+	4+	4+	4+	4+		

* 8% completely inhibited for 96 hours.

* This study was aided by a grant from the Johnson Research Foundation, New Brunswick, N.J.

1 Weinstein, L., and McDonald, A., *Science*, 1945, **101**, 44.

2 Weinstein, L., and McDonald, A., *J. Immunol.*, 1946, **54**, 117.

3 Weinstein, L., and McDonald, A., *J. Immunol.*, 1946, **54**, 131.

4 Lee, S. W., Epstein, J. A., and Foley, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 105.

5 Tsuchiya, H. M., Tenenberg, D. J., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

TABLE II.
Effect of Two Concentrations of Methyl Urea on Varying Amounts of Para-Aminobenzoic Acid in VIB-S.
Inoculum of *E. coli*—S10,000 organisms.

Conc. of methyl urea, %	Sulfanilamide mg/100 ml	*p*-aminobenzoic acid mg/100 ml	Growth (hr) 24	48	72	96
2	—	—	4+	4+	4+	4+
4	—	—	4+	4+	4+	4+
—	15	0.001	0	0	0	3+
—	15	0.0025	0	0	3+	4+
—	15	0.005	0	3+	4+	4+
—	15	0.0075	0	4+	4+	4+
—	15	0.01	2+	4+	4+	4+
—	15	0.025	4+	4+	4+	4+
—	15	0.05	4+	4+	4+	4+
—	15	1.00	4+	4+	4+	4+
—	15	2.50	4+	4+	4+	4+
2	15	0.001	0	0	0	0
2	15	0.0025	0	1+	4+	4+
2	15	0.005	0	3+	4+	4+
2	15	0.0075	1+	4+	4+	4+
2	15	0.01	2+	4+	4+	4+
2	15	0.025	3+	4+	4+	4+
2	15	0.05	4+	4+	4+	4+
2	15	2.50	4+	4+	4+	4+
4	15	0.001	0	0	0	1+
4	15	0.0025	0	0	0	2+
4	15	0.005	0	0	3+	4+
4	15	0.0075	0	1+	4+	4+
4	15	0.01	0	2+	4+	4+
4	15	0.025	0	2+	4+	4+
4	15	0.05	0	2+	4+	4+
4	15	0.75	0	2+	4+	4+
4	15	0.10	0	3+	4+	4+
4	15	2.50	0	3+	4+	4+
—	—	2.50	4+	4+	4+	4+
2	15	—	0	0	0	0
4	15	—	0	0	0	0
—	15	—	0	0	0	0
—	—	—	4+	4+	4+	4+

TABLE III.
Bacteriostatic Effect of Thiourea in Serum-Veal-Infusion Medium.

Organisms. No. in inoculum	hrs	Concentration of thiourea. Growth at various periods: None 48	None 96	2% 48	2% 96	1% 48	1% 96	0.5% 48	0.5% 96	0.25% 48	0.25% 96
E. coli—3250		4+	4+	0	0	0	0	1+	1+	3+	4+
S. aureus—2400		4+	4+	0	0	0	1+	1+	3+	4+	4+

of methyl urea required to suppress growth of *E. coli* was found to be less than that necessary in VIB-S.

A study of the potentiating effect of methyl urea on sulfanilamide in a synthetic medium, using *E. coli* as the test organism, revealed that a 2% concentration of the carbamate produced no apparent increase in activity of

the sulfonamide. Four per cent methyl urea was bacteriostatic when used alone and any stimulation of the activity of sulfanilamide by it could, therefore, not be determined.

II. *The Effect of Methyl Urea on Para-aminobenzoic Acid.* A 2% concentration of methyl carbamate did not inactivate *p*-aminobenzoic acid. Four per cent was moderately effective, the sulfonamide-inhibiting substance being inactivated in amounts as large as 0.1 mg per 100 ml for 24 hours. As with urea and urethane, the anti-PABA activity of methyl urea appeared to be of short duration.

III. *The Bacteriostatic Effects of Thiourea.* A 2% concentration of thiourea inhibited growth of *S. aureus* for 96 hours, 1% for only 48 hours. Quantities less than 1% were ineffective. One per cent thiourea suppressed completely the multiplication of *E. coli.*

Summary and Discussion. Methyl urea was found to be bacteriostatic for several Gram-negative bacteria in both VIB-S and in a synthetic medium. The concentration of drug required to suppress growth varied somewhat with each organism, but it ranged generally between 6 and 8% except for *Ps. aeruginosa*, which was inhibited by a 4% solution. The activity of the drug in a synthetic medium was greater than in VIB-S.

The substitution of one $—NH_2$ in carbamide by a single $—CH_3$ group increased the activity of urea very little since approximately the same concentrations of the 2 compounds were required to inhibit the growth of bacteria. Urethane, as previously reported, was more highly bacteriostatic than either urea or methyl urea. It would appear, therefore, that the insertion of $—CH_2CH_3$ in place of one $—NH_2$ in urea results in greater activity than that following the addition of only a single carbon atom. As shown by the few data presented, the replacement of $=O$ by $—S$ in the urea molecule is apparently very effective in increasing its antibacterial properties since a 1% concentration of thiourea produced approximately the same results with *E. coli* as 3% urethane or 6% urea.

Conclusions. 1. Methyl- and thiourea are bacteriostatic for Gram-negative bacteria. 2. The substitution of an $—NH_2$ group by $—CH_3$ in urea results in no demonstrable increase in antibacterial activity. 3. The replacement of the $=O$ group in urea by $—S$ increases markedly the antibacterial activity. 4. Methyl urea, in the concentrations studied, does not potentiate the sulfonamides. It does have a moderate antagonistic effect on *p*-aminobenzoic acid.

15653

Interrelation Between α-Tocopherol and Protein Metabolism: Body Weight and Tooth Pigmentation of Rats.*

E. L. HOVE. (Introduced by Philip L. Harris.)

From the Research Laboratories of Distillation Products, Inc., Rochester, N. Y.

A relation between vitamin E and protein metabolism has been suggested in 3 reports in the literature. Cerecedo and Vinson[1] observed that a 58% litter incidence of weanling paralysis in mice was reduced to 28% by increasing the casein in the diet of the mothers.

Dam[2] noted that when rats were placed upon a low-protein diet, the group receiving α-tocopherol survived somewhat longer than the E-free controls. However, the *rate* of body weight loss was the same in the 2 groups, and the E-fed group had a lower

* Communication No. 106.

1 Cerecedo, L. R., and Vinson, L. J., *Fed. Proc.*, 1944, 3, 55.

2 Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1944, 55, 55.

body weight at death. Victor and Pappenheimer,[3] on the other hand, reported a very marked effect of tocopherol in conserving the body weight of rats on a low-casein diet. This work is complicated by the fact that a cirrhosis-producing diet (low choline as well as low casein) was used. The effect of tocopherol possibly could have been related to the altered metabolism in the cirrhotic state.

Since these 2 reports appear to be contradictory in some respects, we have repeated the work of Victor and Pappenheimer using equalized feeding of a diet low in casein but otherwise nutritionally adequate.

Fifty-four male weanling rats from the stock colony were placed upon a vitamin E-free diet of the following composition: crude casein, 22.5; sucrose, 63; salt mixture USP No. 2, 4.5; and lard, 10. B-complex vitamins were added to the casein to furnish 10 γ of thiamine, riboflavin and pyridoxine, 25 γ calcium pantothenate, 100 γ niacin, 1 mg of choline chloride, 0.1 mg *i*-inositol, and 5 γ vitamin K per g of ration. Each rat was supplemented weekly with 2,000 units vitamin A and 20 units of vitamin D in olive oil solution.

The animals were kept on this diet, *ad libitum*, until they reached 225 g in body weight. This required 5 to 7 weeks. As they attained this weight the rats were placed on a fixed daily amount of one of 3 diets which differed only in their casein content. These diets contained 5, 20, or 40% crude casein, and, except for compensatory changes in sucrose, were identical in composition with the diet described above.

A daily allotment of 8.9 g of the diet was fed to each rat in the morning and was generally consumed within an hour. The diets were made fresh weekly and kept refrigerated at all times. Half of the rats on each diet were supplemented with daily doses of 1 mg of d, α-tocopherol in olive oil solution.

The average body weight changes of the rats on the 5% casein diet (8.9 g daily) are shown in Fig. 1. These animals lost about 40 g in body weight during the first 4 weeks, after which the curves levelled off. α-Tocopherol had little influence during this period of adjustment to the new diet. Beginning at about the 10th week the vitamin E-low rats began a rapid weight loss. They continued to consume their daily allotment of food in spite of declining body weight. Their general appearance deteriorated rapidly. They became listless and showed marked signs of atony and muscular dystrophy. During the last 3 weeks on experiment 3 of the 9 animals regularly left up to 3 g of their diet unconsumed.

In sharp contrast to the vitamin E-low group, the animals receiving α-tocopherol regained some of their initial weight loss and thereafter maintained their weight. In spite of the low-protein intake their appearance was good. They were alert and showed normal general muscular tone. Statistically the difference in body weight between the E-low and E-fed groups was highly significant. At 21 weeks on experiment the standard error for the average weight of both groups was ± 3.5; this corresponds with a t value of

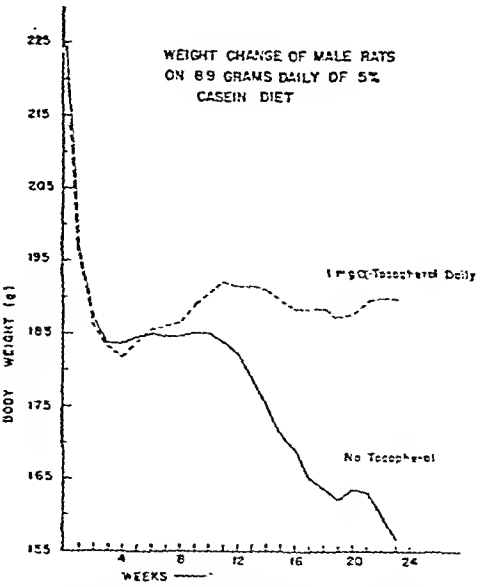

FIG. 1.

The effect of α-tocopherol on body weight changes of adult male rats shifted to a 5% crude casein diet.

[3] Victor, J., and Pappenheimer, A. M., *J. Exp. Med.*, 1945, **82**, 375.

6.2, while for P (0.01) the theoretical t value is 2.95.

At autopsy no signs of liver cirrhosis were evident in either group. The gross appearance and fat content of the livers were normal. Very marked atrophy of the testes was seen in the E-free group; their average testes weight was 0.4 g as compared with an average of 1.2 g in the E-fed group. Eight of the 9 low-E rats had marked hyperplastic crater lesions in the rumen of the stomach, while only 3 of the E-fed rats showed such lesions, and these were mild.

The animals on the higher protein diets required about 2 weeks to adjust themselves to the fixed limited quantity of the new diets. They lost weight during this period. However, after this time they resumed their growth. At the end of 24 weeks the group on the 20% casein diet averaged 292 g while the corresponding E-fed group averaged 303 g. On the 40% casein diet, the average weights were 298 and 308 g for the E-low and E-fed groups respectively. The animals all appeared normal. No signs of dystrophy were yet evident.

An interesting incidental observation has been made on tooth pigmentation in these rats. Granados and Dam[4] have shown that a combination of high-fat and low-E leads to loss of the normal tooth pigment. Our experiment has added protein to the factors influencing this yellow-orange color of the teeth. Table I shows the average color index of the maxillary incisors of the various groups after 10 and 20 weeks on the fixed daily intake of the diets. The color index was rated on an arbitrary scale with 5 degrees ranging from "0" to "4." All of the tocopherol-fed animals had essentially normal tooth color, regardless of protein content of the diet. However, it is clear that the amount of casein greatly influenced the tooth color in the non-tocopherol groups.

TABLE I.
Effects of α-Tocopherol and Protein on Tooth Pigmentation of Adult Rats Fed 8.9 g of Diet Daily. The values represent the average color ratings (0 to 4) of the groups.

Casein content of diet (%)	No tocopherol		Plus tocopherol	
	10th wk	20th wk	10th wk	20th wk
5	0.1	0	3.2	2.8
20	1.4	0.9	3.3	3.2
40	1.6	1.1	3.6	3.2

[4] Granados, H., and Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1945, 59, 295.

Discussion. Under the conditions of this experiment α-tocopherol was essential to the maintenance of body weight and general well-being of adult rats on a low-casein diet. The daily food intake was fixed at a level which, with a normal protein percentage, allowed continued growth and well-being even without tocopherol.

This may indicate that α-tocopherol "spares" or allows better utilization of some constituent of the casein. Conversely, the results may mean that low casein (or some constituent) greatly increases the requirement of the rat for vitamin E. The second explanation appears the more plausible and is currently being checked with rabbits as the experimental animal. Several pure amino acids have been added to low-casein diets at a 1% level in an attempt to find some specific factor which would counteract the dystrophy-inducing property of the low-casein diet. No benefit has been observed with tryptophane, valine, arginine, or lysine. A questionable benefit was seen with *l*-cystine. This work is being continued.

In the work of Dam[2] tocopherol apparently had no influence on the loss in body weight of rats on diets in which the only protein was that furnished by 10% yeast. However, in our work as well as that of Victor and Pappenheimer[3] tocopherol had a marked influence when the only protein was 5% crude casein. The difference in composition of casein and yeast protein may, therefore, furnish a clue as to a specific factor involved in vitamin E metabolism.

Summary. Adult male rats have been fed fixed daily quantities of vitamin E-low diets containing 5, 20, or 40% crude casein. On the 5% casein diet the animals lost weight and developed muscle dystrophy, testicular atrophy, stomach ulcers, and tooth depigmentation.

These symptoms of a vitamin E deficiency were prevented either by α-tocopherol or by

the higher protein diets. The possible significance of a lack of individual amino acids in increasing the vitamin E requirement of rats is discussed.

15654

Induced Ovipositions in Relation to Age of Oviducal Egg in the Domestic Hen.

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The removal of the ruptured follicle from the ovary of the hen results in a delay in the time of lay of the egg which arose from that follicle.[1] The experiments reported here are an approach to the problem of how the ruptured follicle functions to determine the time of lay. Specifically, they were designed to determine whether the sensitivity of the chickens' uterus to an oxytocic agent increases as the time of normally expected lay approaches.

Methods and Materials. Five groups of laying hens were injected intravenously with the same dose of an oxytocic agent at various hours after the ovulation of an egg. The results were expressed as the percentage of birds showing premature ovipositions.

Birds selected for injection carried in their oviducts the second egg of a 2-egg clutch, the first egg of which was laid at the recorded hour of either 10:00 a. m. or 11:00 a. m. The time of lay of all eggs in this laboratory is recorded on the hour every hour between 8:00 a. m. and 4:00 p. m., so that there was a maximum spread of no more than 2 hours between the time of lay of the earliest and latest first egg of a clutch in any group. Of all the birds used, those selected for 10:00 a. m. lay of the first egg of a clutch comprised between 55 and 60% of the total group in 4 groups and 74% of the total in the fifth group.

The presence of the oviducal egg was determined by digital palpation through the rectum. The age of the oviducal egg at the time of injection was estimated from the relationships between time of lay and ovulation of intraclutch eggs,[2,3] and the time required for the egg to pass through the various portions of the oviduct.[2,3] For 2-egg clutches, the interval between lay of the first egg and ovulation of the second is about 45 minutes,[2,3] and the time required for the egg to reach the uterus, about 4 hours from the time it enters the oviduct.[2]

The injections were made during the interval between ovulation and the time of expected lay (a period of about 27 hours) at the following hours: 4:00 p. m., 8:00 p. m., midnight, 6:00 a. m. and 10:00 a. m. Usually, no more than one minute elapsed between the injection of one bird and the injection of the next in each group; the maximum difference between the given times of injection and the time of the earliest or latest injection in the group was 34 minutes. White Leghorn and Rhode Island Red hens were used: the number of one breed did not exceed the number of the other by more than one bird in each group, except in that injected at 6:00 a. m. In this group there were 33 White Leghorns and 12 Rhode Island Reds. The results for the groups as a whole were not appreciably different from the results obtained in each breed.

The agent used to induce premature oviposition was Parke, Davis & Co. "Pitressin." Although the main constituent of this preparation is the pressor principle of the posterior

[1] Rothchild, I., and Fraps, R. M., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 79.

[2] Phillips, R. E., and Warren, D. C., *J. Exp. Zool.*, 1937, 76, 117.

[3] Unpublished data from this laboratory.

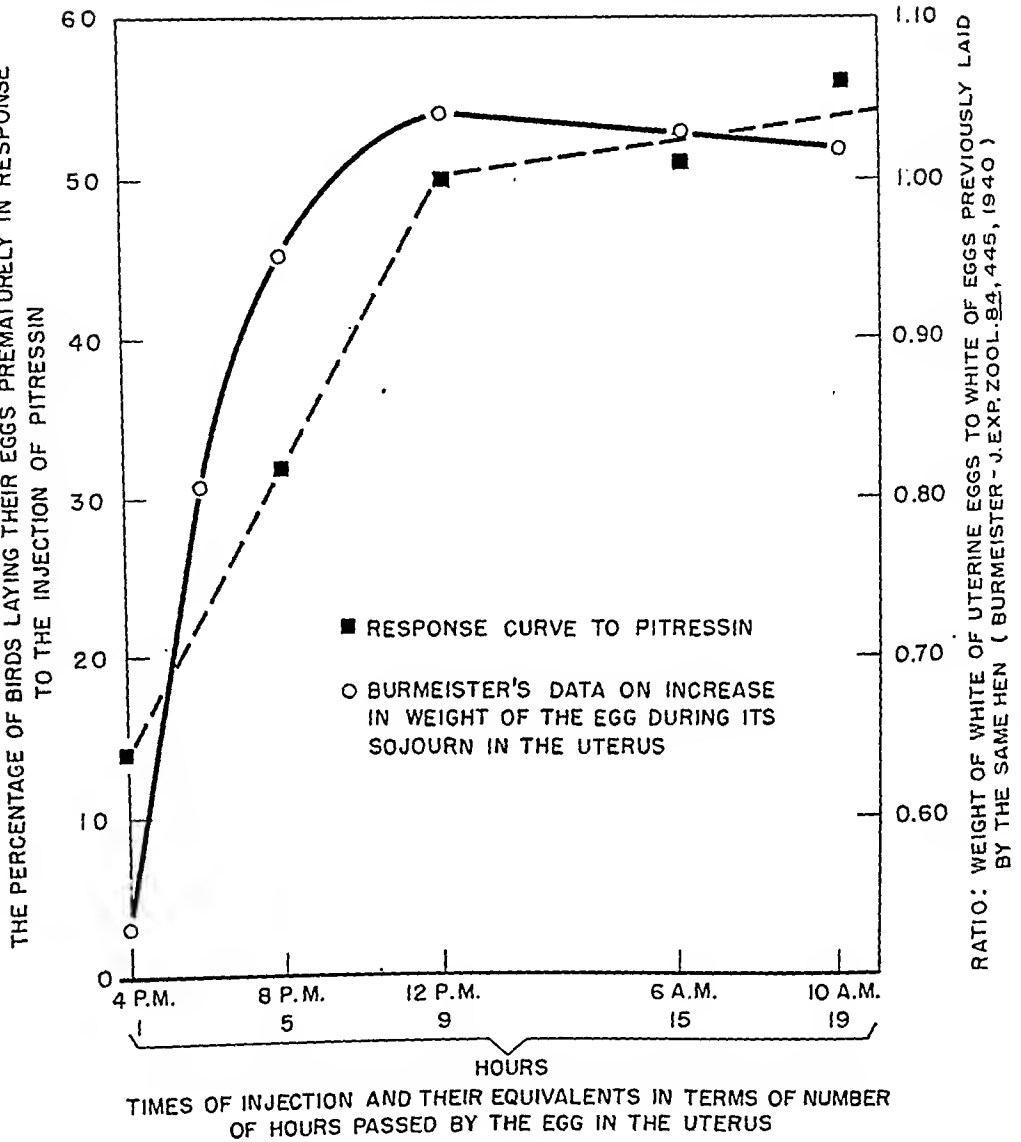

FIG. 1.

Comparison of the curve showing percentage response to Pitressin in relation to the age of the oviducal egg with the curve relating increase in size of the egg to the length of time spent by the egg in the uterus.

pituitary, it has been shown by Burrows and Fraps[4] to have a strong oxytocic action in the chicken. The dose used was 0.30 unit of labeled potency; this was prepared by diluting 1.0 cc, taken from a separate 1.0 cc vial, and containing 20.0 pressor units, to 10.0 cc with 0.9% NaCl. The amount injected was 0.15 cc. A diluted preparation was never used for more than 2 days in succession. The same 0.5 cc hypodermic syringe was used for all injections.

A premature oviposition in response to Pitressin was taken as lay of the oviducal

[4] Burrows, W. H., and Fraps, R. M., *Endocrinology*, 1942, **30**, 702.

TABLE I.
Relation Between the Percentage of Birds Reacting by Premature Oviposition to a Constant Dose of Pitressin (0.30 Unit) and the Hours After Ovulation That the Injection Was Made.

Injections		Birds injected		
			Positive reactions*	
Time	Approx. No. hr after ovulation	No.	No.	%
4 p.m.	5	22	3	14
8 "	9	31	10	32
12 "	13	44	22	50
6 a.m.	19	45	23	51
10 "	23	32	18	56

* A positive reaction is the expulsion of the oviducal egg in response to the injection of Pitressin within 30 minutes after injection.

egg within a period of 30 minutes from injection. Practically all premature ovipositions, however, occurred well within 15 minutes from the time of injection.

Results are shown in Table I. Between 4:00 p. m. and midnight following the ovulation of these particular eggs, the percentage of premature ovipositions in response to 0.30 unit of Pitressin increased substantially. From midnight to 10:00 a. m. the next day, however, there was only a slight, if at all significant further increase in the percentage of premature ovipositions. Since 10:00 a. m. was only 2 to 6 hours from the time of normally expected lay for these particular eggs, it can probably safely be assumed that there was very little change in the response to a given dose of Pitressin from midnight until the actual time of lay.

Removal of the ruptured follicle apparently did not affect the percentage of birds responding by premature oviposition to this dose of Pitressin. Of 17 birds so treated, 9, or 53%, laid prematurely. This compares very well with the group of intact birds injected at the same hour. This fact, together with the evidence of a plateau from midnight on, indicated that the response curve might be a manifestation of the mechanical stretching of the uterine walls as the egg increases in size (plumping) with the uptake of water.[5] To check this, Burmeister's data[5] on the increase in weight of the egg in relation to time spent in the uterus was compared with the response curve to Pitressin. The 2 curves are shown in Fig. 1. For comparison, the hour of injection was made equivalent to time spent in the uterus by means of the following rough calculations. The hour of lay of the first egg of the clutch for each group as a whole was taken as 10:00 a. m. (This probably errs somewhat in the direction of a later-than-actual average, but not enough to affect appreciably the overall picture). Ovulation of the test egg would therefore have occurred about 10:45 a. m., and the egg would have entered the uterus somewhere around 2:45 p. m.-3:00 p. m. (see above). The 2 curves were plotted, taking 3:00 p. m. as the 0 point for time spent in the uterus. The response curve shows the beginning of a plateau very close to the time that the egg becomes fully plumped, *i.e.*, reaches its almost maximum size. The only further increase in size that takes place from this point on is that due to shell formation (which is not shown by Burmeister's[5] figures), and it may well be that the slight increase in the percentage response to Pitressin during the period of shell formation is a reflection of this phenomenon.

Discussion. The increasing percentage of birds responding to a given dose of Pitressin as their eggs increase in size, and the lack of any substantial further increase in response after the egg reaches its almost maximum size, may be taken to indicate that the changes in reactivity of the uterus during the sojourn in it of the egg are not directly responsible for the determination of the time of normal oviposition. With the reservation that a response to Pitressin is only a probable indication of the sensitivity of the uterus to the stimulus which under normal conditions leads to oviposition, it may be assumed for the present, therefore, that the probable mode of action of the ruptured follicle is one that primarily involves a change in some system external to the uterus.

Summary. Hens carrying oviducal eggs were injected with 0.30 unit of Pitressin at times equivalent to 1, 5, 9, 15, and 19 hours of time passed by the egg in the uterus. The percentages of birds laying prematurely were respectively 14, 32, 50, 51, and 56.

[5] Burmeister, B. R., *J. Exp. Zool.*, 1940, **84**, 445.

Removal of the ruptured follicle shortly after ovulation did not alter the response at the 19th hour in the uterus stage. The beginning of the plateau of the response curve approximately coincided with the time at which the oviducal egg became fully plumped, *i.e.*, reached its almost maximum size. The response curve was interpreted as the expression of a change in the mechanical relations between the uterus and the contained egg, rather than a change in intrinsic sensitivity due to the action of the ruptured follicle.

15655

Physiological Disposition of Penicillin G and K in Dogs.

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The unexpected low activity of penicillin K in experimental syphilis first observed by Chesney[1] has been confirmed.[2] In addition it has been demonstrated that penicillin K is less effective than G in the treatment of mice infected with pneumococcus Type I,[3] *Streptococcus pyogenes*[3] and *Borrelia novyi*.[4,5]

This low activity of penicillin K has been correlated with an apparently low plasma concentration and it has been suggested that this is a result of more rapid destruction in the animal body.[3,6] A more detailed study of the disposition of penicillin G and K in dogs has led us to a somewhat different conclusion, and has cast considerable doubt on the supposition that inactivation plays a major part in the immediate fate of injected penicillin K.

Recovery of Added Penicillin G and K from Body Fluids and Tissues. The recovery of added penicillin from various biological materials was studied by the customary cup test procedure of assay for penicillin concentration. Known amounts of each penicillin* were added to body fluids and to tissue mashes, and the actual assay figures in each case were related to the assay figures of the same concentration of penicillin added to saline.

Plasma was obtained by centrifuging oxalated or heparinized dog blood. Tissue mashes were prepared with a homogenizer, using 2 parts of saline for one of muscle, and equal parts of saline and tissue in all other cases. The grinder was immersed in ice water during the preparation of each tissue. One-tenth of a volume of an appropriate saline solution of penicillin was added to a series of samples of tissue mashes, plasma, and saline. Final concentrations of penicillin ranged from 0.3 μg per ml to 24 μg per

1 Unpublished observations quoted by Eagle and Musselman, *Science*, 1946, **103**, 618.

2 Rake, G., Dunham, W., and Donoviek, R., to be published.

3 Eagle, H., and Musselman, A., *Science*, 1946, **103**, 618.

4 Burk, M., Farr, A. C., and Schnitzer, R. J., *Science*, 1946, **104**, 370.

5 Richardson, A. P., Loeb, P., and Walker, H. A., unpublished observations.

6 Coghill, R. D., Osterberg, A. E., and Hazel, G. R., *Science*, 1946, **103**, 709.

* We are indebted to Drs. Wintersteiner and Adler of the Division of Organic Chemistry, and Mr. Lott and Mr. Dolliver of the Division of Medicinal Chemistry for these preparations. Elementary analysis and bioassay values for both penicillins agreed well with theoretical values. Analysis by Craig distribution methods showed the G preparation to be at least 92% homogeneous, the remainder consisting of inactive components. The K preparations were at least 88% of "K type" penicillins, not more than 10% of an active fraction which was probably an "F type" penicillin, and the remainder was an inactive component.

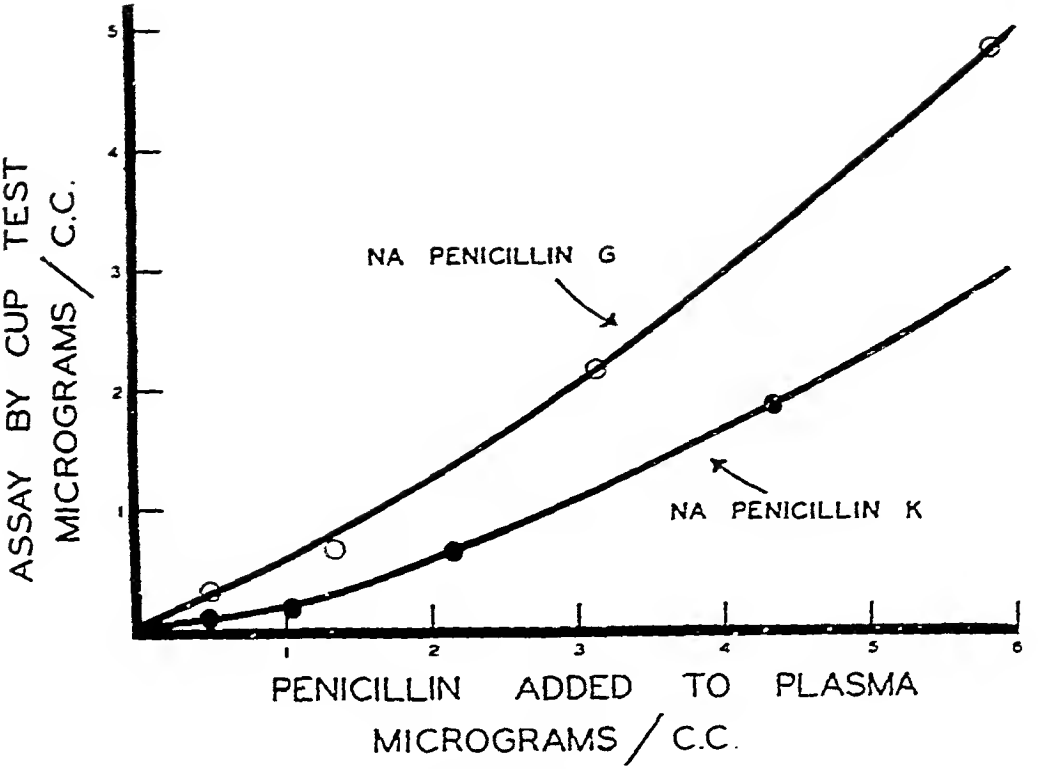


FIG. 1.

Recovery of Penicillin G and K from dog plasma, when assayed by the cup test procedure. Each curve represents an average of six experiments carried out on different days.

ml. The assays were then carried out by the usual cup test procedure.*

The recovery of penicillin added to saline was within 10% of the predicted concentration in all cases. With all tissues and plasma, the recoveries were incomplete and the extent of the loss was inversely proportional to the concentration of penicillin. Fig. 1 summarizes a portion of 6 typical experiments obtained with plasma, and is presented because it shows a marked difference in behavior of the 2 penicillins when added to this body fluid. It is quite obvious that cup test assays of penicillin K in plasma are subject to considerable error, unless a correction is made for incomplete recovery. Proof that the incomplete recoveries were due to the assay procedure used and not due to destruction of penicillin K was obtained by 2 experiments.

1. Simultaneous assay of representative samples of penicillin-plasma mixtures by the tube dilutions technics gave complete recoveries. For example, in one experiment in which 1.25 μg per ml of K was added to plasma, cup test assays gave 20% recovery, whereas tube dilutions assays gave 100%

TABLE I.

Average Per Cent Recovery of Penicillin G and K from Dog Plasma and Tissue Mash by Cup Test at Different Concentrations.

	5 γ/ml		1 γ/ml	
	G	K	G	K
Plasma	83.0	42.5	65.0	17.1
Liver	63.5	64.5	45.0	28.2
Kidney	62.5	55.5	35.0	25.0
Spleen	75.0	50.0	45.0	42.0
Brain	87.0	78.0	60.0	37.5
Muscle	73.0	80.0	60.0	54.0
Lung	54.0	66.0	45.0	44.0

* Bioassays reported in this study were carried out under the general supervision of the Division of Microbiology.

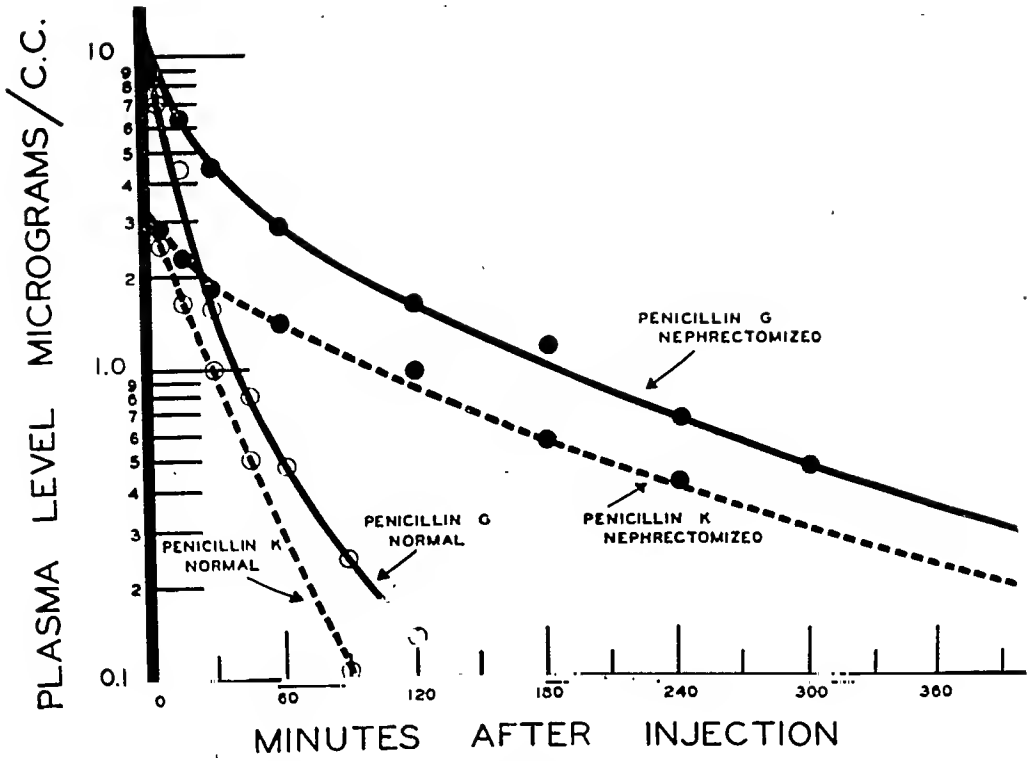


FIG. 2.

The rate of disappearance of penicillin G and K from the plasma of dogs following intravenous injection of 2 mg per kilo. Each curve is the mean of experiments in 6 dogs. All figures have been corrected for error in the cup test assay.

recovery.

2. Incubation of plasma-penicillin mixtures for 3 hours at 22°C or 24 hours at 4°C gave the same recovery as when the penicillin was added immediately preceding assay. Thus if destruction had been responsible for the low cup test assay it must have occurred immediately and then stopped within a few minutes.

The results of all recovery experiments with plasma and tissues are summarized in Table I. The per cent recovery for each material was obtained by plotting added penicillin against assay results in a form similar to Fig. 1. Per cent recovery of the 2 concentrations was determined from the curves thus obtained. All results reported later in this paper have been corrected by the use of such curves to give "true" penicillin concentration.

Rate of Disappearance of Penicillin G and K from the Plasma of Normal and Nephrectomized Dogs. The rate of disappearance of a drug from the plasma of an intact animal is the net result of distribution, excretion and destruction. In the case of penicillin it is well recognized that rate of disappearance is conditioned largely by renal excretion. Therefore, the rate of fall of plasma-penicillin in a nephrectomized dog should reflect distribution, destruction and extrarenal excretion.

Experiments were set up as follows: each dog was injected intravenously with 2 mg per kg of the appropriate penicillin. Blood samples were taken at short intervals of time until the plasma concentration approached zero. The following day a nephrectomy was performed, and after 24 hours recovery, the same dose of the same penicillin was injected intravenously and rate of disappearance from plasma again determined. Six

TABLE II.
Distribution of Penicillin G and K Between Plasma and Tissues of Dogs Following Continuous Intravenous Injection.

Dog No.	Plasma level, γ/ml		Plasma-tissue ratios after 2 hrs infusion						
	After 1 hr Infusion	After 2 hr Infusion	Cerebrospinal Fluid	Brain	Muscle	Spleen	Lung	Liver	Kidney
			Penicillin G—0.1 mg/kilo/minute.						
25	13.8	23.5	151.0	47.0	5.2	5.9	2.1	3.3	0.25
26	15.0	24.7	185.0	62.0	5.1	9.5	2.0	2.7	1.1
27	11.2	16.0	191.0	32.0	4.4	3.8	1.4	3.6	0.42
28	17.3	14.6	91.0	18.4	6.1	6.1	2.1	1.4	0.43
29	24.3	28.1	220.0	56.0	5.9	4.5	2.7	2.7	0.64
30	15.1	16.7	230.0	69.0	10.6	5.2	2.4	1.9	0.46
Mean	16.1	20.6	176.0	47.4	6.2	5.8	2.1	2.4	.55
			Penicillin K—0.2 mg/kilo/minute.						
31	12.5	18.3	62.0	18.3	4.7	4.6	2.7	1.8	0.55
32	17.5	24.8	220.0	20.6	8.3	9.6	4.8	1.9	0.91
33	13.6	13.5	151.0	34.0	6.1	9.1	3.2	1.1	0.52
34	12.6	13.5	135.0	22.5	6.4	8.5	3.8	1.2	0.81
35	19.5	27.0	270.0	45.0	*	*	*	1.2	0.82
Mean	15.1	19.4	167	28.1	6.4	7.9	3.8	1.4	0.72

* Not determined.

dogs were used for each penicillin. Scrutiny of the data (Fig. 2) shows a number of points of interest:

1. In as short a time as 5 minutes after injection the plasma level of penicillin K is less than one-half that seen when the same dose of G was injected. This finding suggests that K is either destroyed within 5 minutes or some other factor is responsible for the disposition of K as compared to G.

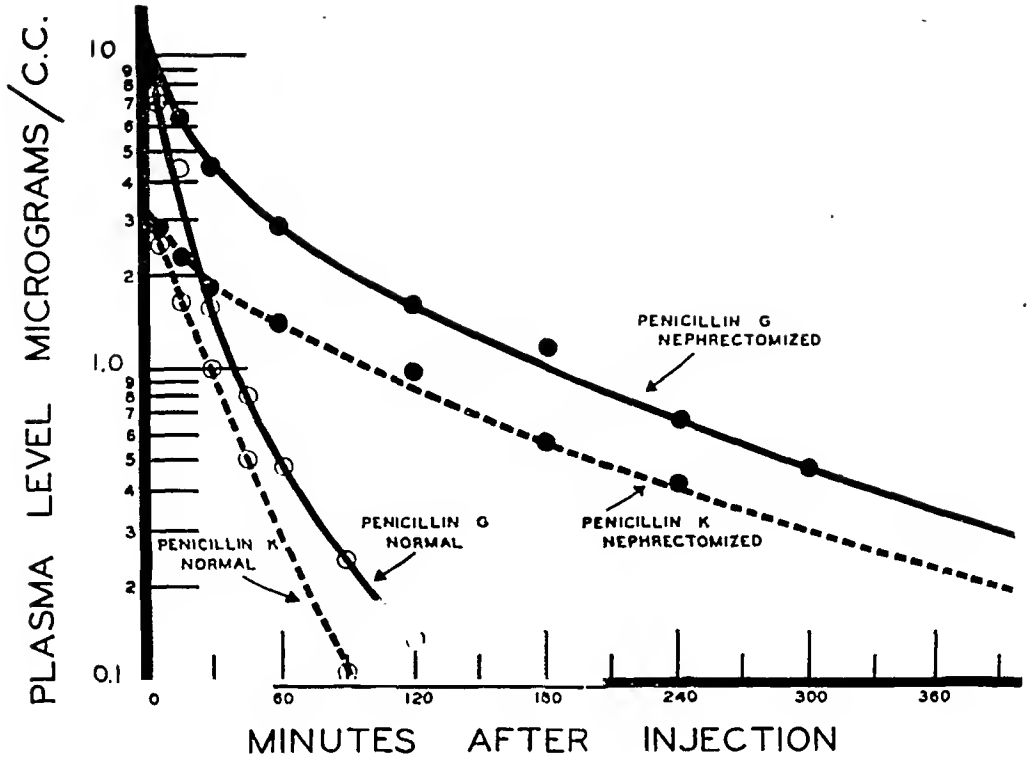
2. From a given plasma level the rate of disappearance of the 2 penicillins is not greatly different. If destruction had been a major factor in determining the fate of one penicillin as compared to another, the slope of these curves should have been different. Such was not the case.

3. Extending the time-plasma level curve back to zero permits a calculation of the apparent volume of distribution of each penicillin. For G this was estimated as 20.2% or corresponds roughly to total blood volume plus extra-cellular tissue fluid. In the case of penicillin K the apparent volume of distribution was 57.1% which at once suggested localization in tissues or some body fluid, other than blood.

Distribution of Penicillin G and K in Body

TABLE III.
Distribution of Penicillin G and K Between Dog Plasma and Saline After 20 Hr Dialysis at 4°C.

Concentration added to saline γ/ml	No. of exp.	Mean concentration after dialysis—γ/ml		Apparent % "bound"
		Plasma	Saline	
		Na Penicillin G.		
3.0	8	3.6	2.3	46.7
30.0	6	46.3	28.6	36.1
		Na Penicillin K.		
2.3	9	3.6	1.0	71.0
23.0	6	39.1	11.2	70.6



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Summary. 1. As determined by the customary cup test procedure it is not possible to obtain complete recoveries of penicillin G and K when added to plasma and tissues. A considerable error is involved in the assay of penicillin K in the presence of a high concentration of plasma. Such low recoveries have been shown not to be due to destruction of penicillin K. 2. For a given dose, the plasma level of penicillin K 5 minutes after intravenous injection is less than one-half that with penicillin G. The rate of disappearance from a given plasma concentration, however, is not greatly different for the 2 penicillins. 3. The apparent volume of distribution of G is 20.2% of the body weight, whereas with K it is 57.1%. Both of the penicillins are localized in lung and liver to some extent. Penicillin K is localized to a considerably greater extent in the liver than is G. 4. As determined by dialysis experiments penicillin K is adsorbed by some component of dog plasma to a much greater extent than is penicillin G.

15656

Subtilin-Antibiotic Produced by *Bacillus subtilis*.* V. Effect on *Streptococcus pyogenes* Infections in Mice.†

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From the Department of Bacteriology, University of California, Los Angeles.

In a previous communication[1] subtilin was found to exert a pronounced antibiotic action against a large number of species of Gram-positive organisms, including *Streptococcus pyogenes* (hemolytic) by the agar cup-plate procedure. The agent was shown to be bacteriostatic in high dilution and bactericidal in more concentrated solution. Subtilin exhibited an extremely low toxicity index to living embryonic chick heart tissue fragments cultivated *in vitro*[2] and exerted a powerful *in vivo* action on the course of experimental pneumococcus infections in mice[3] and anthrax in guinea pigs.[4]

TABLE I.
Treatment of *Streptococcus pyogenes* Infections in Mice.

		6 mice used in each group				
Date	Time	Control	Treated immediately	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
8-13-46	9 a.m.	Infected	Infected	Infected	Infected	Infected
"	9 "	—	0.5 cc subtilin	—	—	—
"	12 m.	—	" " "	0.5 cc subtilin	—	—
"	3 p.m.	—	" " "	" " "	0.5 cc subtilin	—
"	6 "	—	" " "	" " "	" " "	0.5 cc subtilin
"	11 "	All dead	1.0 " "	1.0 " "	1.0 " "	1.0 " "
14	9 a.m.	—	" " "	" " "	" " "	" " "
"	12 m.	—	" " "	" " "	" " "	" " "
"	4 p.m.	—	" " "	" " "	" " "	" " "

* This investigation was aided by a grant from Eli Lilly and Co., Indianapolis, Indiana.

† The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, Calif.

1 Salle, A. J., and Jann, Gregory, J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 60.

2 Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 23.

3 Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 40.

4 Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 41.

Fluids and Tissues After Continuous Infusion. Since calculation of the apparent volume of distribution of penicillin K suggested localization, it was obviously necessary to determine the distribution of the 2 penicillins in tissues. There are only a few data available in the literature on the distribution of penicillin in tissues of animals, and in all cases the studies[7,8] have been carried out with impure preparations. In our studies dogs were lightly anesthetized with sodium pentobarbital following which a cannula was inserted into a femoral vein and attached to a burette, so that an appropriate concentration of penicillin could be infused at a constant rate of 1 ml per minute. Plasma concentration was determined at the end of one hour and again at 2 hours, following which infusion was discontinued, the animal was killed and representative tissues removed and prepared for assay as described above. Each penicillin was administered in a dose which was calculated to maintain the plasma concentration between 15-20 μg per ml for the period of infusion so as to obtain equilibrium between plasma and tissues. To accomplish this, the rate of infusion of G was maintained at 0.1 mg per kg per minute. For K, 0.2 mg per kg per minute was required.

Results of these experiments are summarized by Table II. As was expected kidney had the highest concentration, whereas brain and spinal fluid had the lowest levels. Lung and liver had higher concentrations than were predicted on the basis of blood and tissue fluid content, thus suggesting some localization. The most striking difference in distribution of the 2 penicillins was noted in the liver assays. The concentration of K in this organ approached the concentration in plasma, and was nearly twice as high as that of G.

Adsorption of Penicillin G and K by Dog Plasma. The error in cup test assays of penicillin K in plasma described above indicated the likelihood of binding by some constituent of plasma. A cellophane bag containing 7.5 ml of plasma was suspended in a dialysis tube and equilibrated with 15 ml of saline containing a desired concentration of penicillin. Dialysis was carried out on a rocking machine at 4°C for 20 hours, following which penicillin concentration in each fluid was determined.

The results summarized in Table III show that in all instances the concentration of penicillin in plasma was higher than in the corresponding saline solution. Per cent of "bound" penicillin was calculated by the usual method of subtracting concentration in saline from concentration in plasma and dividing by total plasma concentration. Interpretation of the data is difficult because of an apparent high recovery of both penicillins in these experiments, which could not be explained by irregularities in the assay procedure. In spite of this there is little question but that K was bound to a far greater extent than G; thus furnishing a reasonable explanation for the difficulty of determining true concentration of penicillin K in plasma by the cup test procedure.

Discussion. The foregoing experiments leave little doubt as to the fact that penicillin K is handled by the intact animal in a quantitatively different manner than G. The fundamental basis for these differences is not clear at the moment in terms of the commonly recognized chemical properties of the 2 penicillins. However, data on the physical characteristic of these penicillins are not now available, and it may be that information along these lines will clarify the problem.

Regardless of the explanation of the differences in properties of these 2 penicillins, K has an apparent disadvantage for the treatment of systemic infections. This arises from the greater difficulty in maintaining a given total plasma concentration, and because it is likely that adsorption decreases the effective plasma level. Cup test assays may give a misleading picture of total penicillin K content in plasma. However, they may well give a truer indication of the concentration of "free" penicillin available for therapeutic effect.

[7] Stuble, G. G., and Bellows, J. G., *J. A. M. A.*, 1944, **125**, 685.

[8] Cutting, W. C., Luduena, F. P., Fiese, M., Elliot, H. W., and Field, J., *J. Pharm. and Exp. Therap.*, 1945, **83**, 36.

Summary. 1. As determined by the customary cup test procedure it is not possible to obtain complete recoveries of penicillin G and K when added to plasma and tissues. A considerable error is involved in the assay of penicillin K in the presence of a high concentration of plasma. Such low recoveries have been shown not to be due to destruction of penicillin K. 2. For a given dose, the plasma level of penicillin K 5 minutes after intravenous injection is less than one-half that with penicillin G. The rate of disappearance from a given plasma concentration, however, is not greatly different for the 2 penicillins. 3. The apparent volume of distribution of G is 20.2% of the body weight, whereas with K it is 57.1%. Both of the penicillins are localized in lung and liver to some extent. Penicillin K is localized to a considerably greater extent in the liver than is G. 4. As determined by dialysis experiments penicillin K is adsorbed by some component of dog plasma to a much greater extent than is penicillin G.

15656

Subtilin-Antibiotic Produced by *Bacillus subtilis*.* V. Effect on *Streptococcus pyogenes* Infections in Mice.†

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In a previous communication[1] subtilin was found to exert a pronounced antibiotic action against a large number of species of Gram-positive organisms, including *Streptococcus pyogenes* (hemolytic) by the agar cup-plate procedure. The agent was shown to be bacteriostatic in high dilution and bactericidal in more concentrated solution. Subtilin exhibited an extremely low toxicity index to living embryonic chick heart tissue fragments cultivated *in vitro*[2] and exerted a powerful *in vivo* action on the course of experimental pneumococcus infections in mice[3] and anthrax in guinea pigs.[4]

TABLE I.
Treatment of *Streptococcus pyogenes* Infections in Mice.

		6 mice used in each group				
Date	Time	Control	Treated immediately	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
8-13-46	9 a.m.	Infected	Infected	Infected	Infected	Infected
"	9 "	—	0.5 cc subtilin	—	—	—
"	12 m.	—	" " "	0.5 cc subtilin	—	—
"	3 p.m.	—	" " "	" " "	0.5 cc subtilin	—
"	6 "	—	" " "	" " "	" " "	0.5 cc subtilin
"	11 "	All dead	1.0 " "	1.0 " "	1.0 " "	1.0 " "
14	9 a.m.	—	" " "	" " "	" " "	" " "
"	12 m.	—	" " "	" " "	" " "	" " "
"	4 p.m.	—	" " "	" " "	" " "	" " "

* This investigation was aided by a grant from Eli Lilly and Co., Indianapolis, Indiana.

† The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, Calif.

1 Salle, A. J., and Jann, Gregory, J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 60.

2 Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 23.

3 Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 40.

4 Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 41.

TABLE II.
Treatment of *Streptococcus pyogenes* Infections in Mice.

Date	Time	8 mice used in each group				
		Control	Treated immediately	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
9-19-46	9 a.m.	Infected	Infected	Infected	Infected	Infected
"	9 "	—	0.5 cc subtilin	—	—	—
"	12 m.	—	" " "	0.5 cc subtilin	—	—
"	3 p.m.	—	" " "	" " "	0.5 cc subtilin	—
"	6 "	—	" " "	" " "	" " "	0.5 cc subtilin
"	8 "	1 dead	—	—	—	—
"	9 "	—	1.0 cc "	1.0 " "	1.0 " "	1.0 " "
9-20-46	9 a.m.	All dead	0.5 " "	0.5 " "	0.5 " "	0.5 " "

In the present communication subtilin was tested for its effect on *Streptococcus pyogenes* infections in white mice.

Experimental. Thirty white mice, weighing between 20 and 25 g were injected intraperitoneally with 0.2 cc of a saline suspension of a 24-hour brain heart infusion agar slant culture of *Streptococcus pyogenes*. The turbidity of the saline suspension was adjusted to correspond to a No. 3 MacFarland nephelometer standard.

The animals were divided into 5 groups and treated as follows: mice in Group 1 were not treated but served as controls; mice in Group 2 were treated immediately after infection; mice in Group 3 were treated 3 hours after infection; mice in Group 4 were treated 6 hours after infection; and the mice in the last group were treated 9 hours after infection. Each mouse was injected intraperitoneally with 0.5 cc of a solution containing 0.2 mg subtilin per cc (1 unit)[2] and every 3 hours thereafter. This dose was increased to 1 cc (2 units) at the end of the first day and throughout the next.

The schedule of treatments and results obtained are given in Table I. It may be seen that all of the control animals died within 14 hours whereas all of the treated animals survived and were apparently normal 2 weeks after treatment was discontinued. The mice were discarded after this period of time.

The animals treated 9 hours after being infected were 5 hours from death and appeared to be beyond recovery. The infection was accompanied by diarrhea, ruffled fur, and prostration. After receiving 2 injections of subtilin, the diarrheal condition disappeared and the animals appeared to be almost normal. After the fourth injection, the animals looked and acted like healthy mice and devoured food freely. The recovery was so spectacular that it was almost beyond belief.

In a second series a larger number of mice (40) was used to check the results in the first experiment. The method was the same but the amount of subtilin administered was less. The results are recorded in Table II.

It may be seen that one of the untreated mice died within 11 hours. The others died within 24 hours but the exact time could not be ascertained because death occurred sometime during the first night. On the other hand, all of the animals given subtilin survived and were healthy and normal 2 weeks after treatment was discontinued. The animals were sacrificed after this period of time. Examination of the hearts' blood of animals dead of the disease showed the presence of large numbers of *Streptococcus pyogenes*. The antibiotic did not produce any observable toxic reaction in the mice.

Conclusions. Subtilin has been shown to exert a powerful *in vivo* action on the course of experimental *Streptococcus pyogenes* infections in mice. Animals given subtilin 9 hours after being infected were in such an advanced stage of the disease at the time treatment was started that recovery was almost unbelievable. The antibiotic exhibited no apparent toxic reaction in the mice.

15657 P

Biotin Deficiency in the Dairy Calf.*

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During the course of the experiments on the development of a synthetic ration for the dairy calf (described elsewhere),[1] 2 of the animals developed a paralysis that was cured by the administration of biotin. This syndrome occurred when the animals were fed Ration No. 2. The composition of the diet is given in Table I.

The lard was homogenized into the solution of casein, salts and cerelose to produce a "synthetic milk" which contained 13% solids. All vitamin supplements were given at the morning feeding. For complete details on the preparation of the diet see Wiese, Johnson, Mitchell and Nevens.[1]

The first animal, calf No. 7, having been on Ration 2 for 19 days, developed a paralysis of the rear legs and was unable to rise. The calf, very unsteady when placed on his feet, was incapable of muscular coordination of the hind legs and fell when trying to walk.

An attempt was made to cure the condition by giving the animal 25 g of Wilson's 1-20 liver powder in the morning feeding. A slight improvement was shown by afternoon. The following morning, the paralysis had disappeared, and the calf was able to rise without assistance and walk normally. The animal, continued on Ration 2, exhibited the same paralytic symptoms 22 days later. The calf was given 100 μg of biotin by subcutaneous injection. In 24 hours the symptoms had disappeared and the animal had recovered completely.

Calf No. 7 developed the identical symptoms a third time 13 days later and was cured again by the subcutaneous injection of 100 μg of biotin.

A second animal, calf No. 9, having been on the diet for 50 days, showed the same symptoms as calf No. 7. The calf was given 100 μg of biotin by subcutaneous injection. When the condition of the animal did not improve, 1 mg of biotin in glucose was administered by intravenous injection. The next day the animal had recovered completely.

TABLE I.
Composition of Ration 2.

Component	%	Vitamin added	Mg/kg liquid ration
Casein "Labco"	30.0	Thiamin	0.65
Lard	26.6	Riboflavin	0.65
Salts 1	6.0	Nicotinic acid	2.60
Cerelose	37.4	Pyridoxine	0.65
		Calcium pantothenate	1.30
		Ascorbic acid	13.0
		α-Tocopherol	1.0
		2-Methyl-1,4-naphthoquinone	0.26
		Vitamin A	5000 I.U./day
		Vitamin D	500 I.U./day

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† Present address: Department of Agricultural Chemistry, University of Idaho.

[1] Wiese, A. C., Johnson, B. Connor, Mitchell, H. H., and Nevens, W. B., *J. Dairy Sci.*, in press.

TABLE II.
Urinary Excretion of Biotin.

Date	Symptoms	Treatment	Excretion γ/day
		Calf 7.	
Born 1-28-46			
2-12	—		5.2
13	—		4.4
17	+		
18	+	25 g liver	—*
19	—		
3-12	+	100 γ biotin	—*
13	—		
25	+	100 γ biotin	—†
26	—		
4-1	—	On starch containing diet	
5-4	—		14.6
29	—		10.6
		Calf 11.	
Born 4-24-46			
4-26	—	On colostrum	46.4
5-3	—	On synthetic milk diet	4.4
4	—		5.3
9	—		2.5
10	—	+ biotin†	5.7
18	—		7.3
24	—		7.6
29	—		10.6
6-4	—		14.3

Date	Symptoms	Treatment	Excretion γ/day
		Calf 9.	
Born 2-12-46			
2-23	—		1.9
24	—		3.1
4-5	+	100 γ biotin	—*
6	—		
18	—	On starch containing diet	10.5
19	—		15.1
5-17	—		25.6
29	—		10.7
6-6	—		21.2
		Calf 12.	
Born 4-24-46			
4-26	—	On colostrum	35.1
5-3	—	On synthetic milk diet	2.1
4	—		3.9
9	—		3.2
10	—	+ biotin†	8.2
18	—		5.4
24	—		4.8
6-1	—		12.6

* Urine samples not obtained due to scouring.
† Biotin added to the "synthetic milk" diet at the rate of 10 μg per 1000 cc of milk.

Calves No. 7 and 9 were changed to a solid diet containing considerable amounts of starch when 7 weeks old. On this diet the animals did not exhibit any paralytic symptoms.

During the course of the experiment 24-hour urine collections were made. The amount of biotin excreted was determined by the method of Wright and Skeggs.[2] The data are given in Table II. At the age of 2 weeks, calves No. 7 and 9 excreted only a small amount of biotin in the urine. The increase in biotin excretion by these animals during the latter stages of the experiment is probably due to the change to a solid diet containing considerable amounts of starch. On this type of ration intestinal synthesis of the B-vitamins, including biotin, probably occurred, accounting for the increased biotin excretion in the urine.

Calves No. 11 and 12 were maintained on the liquid "synthetic milk" diet. Biotin was not supplemented for the first 2 weeks. After this time 10 μg of biotin were added per kg of liquid ration fed. The first urine sample was obtained when the calves were 48 hours old and had received colostrum. The data in Table II reveal that the biotin excretion was high at this time. After one week on the biotin-deficient ration the urinary biotin excretion had dropped to 3 to 5 μg per day. After the supplementation of biotin was begun, there was a gradual increase in the urinary excretion of biotin. These 2 calves grew normally and did not show any symptoms of biotin deficiency during the time they were on the experiment.

Summary. The young dairy calf requires an exogenous supply of biotin; otherwise a paralysis of the hind quarters develops, curable by biotin administration.

2 Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 95.

15658

Changes Produced by Starvation in the Vitamin Content of Rat Tissues.

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(Introduced by Ralph R. Mellon.)

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It has been observed in this laboratory that the topical application of β,β dichloroethyl sulfide (mustard gas) in rats causes marked changes in the vitamin content of some tissues. Since severe starvation is a prominent feature of mustard gas poisoning, it was deemed necessary to determine the effect of starvation *per se*. This report describes the effects of mustard gas and severe starvation upon the liver, kidney, and bone marrow contents of 6 of the B vitamins.

Methods. Animals. Twenty-five-day-old, male, albino rats of the Sprague-Dawley strain were housed in individual wire-mesh cages and were fed the following ration: casein (Labco, "vitamin-free") 18; sucrose, 76; salts IV,[1] 4; and corn oil, 2. To each kilogram of ration were added: thiamine hydrochloride, 3.0 mg; riboflavin, 3.0 mg; pyridoxine hydrochloride, 3.0 mg; calcium pantothenate, 10.0 mg; *i*-inositol, 0.3 g; choline chloride, 1.0 g; and 2-methyl-1,4-naphthoquinone, 10.0 mg. At the beginning of the experiment, the rats were given one drop of halibut liver oil (Abbott-plain) containing 1.3 mg of added *dl*-α-tocopherol acetate. All of the rats received this ration *ad libitum* for 4 days, at which time the experimental procedures were initiated.

Tissues. At the conclusion of the experiments the rats were decapitated, and the tissues removed for analysis. The total weights of the livers were determined, and weighed portions of both the liver and kidney were dried for 48 hours at 45°C *in vacuo*. The samples were reweighed to determine the dry weights, then stored in the cold until prepared for analysis. The dried livers and kidneys were ground and a weighed portion (approximately 50 mg) homogenized in 10 ml of water with a glass homogenizer.[2] Each sample was incubated at 37°C under toluene with clarase and papain (each enzyme added at a level equivalent to 5% of the weight of the tissue) for 24 hours at the natural pH, and then for 24 hours in .05 N sodium acetate buffer at pH 4.5.

The 6 long bones (femora, tibiae, and humeri) were split with a scalpel and the marrow, which was removed with a hypodermic needle filed on one side to form a scoop, was suspended in water and stored in the cold under toluene until prepared for assay. The bone marrow was homogenized and the nitrogen content determined on an aliquot by a micro digestion and nesslerization procedure.[3] One gram of each of the enzymes per gram of nitrogen was added and the tissue digested as above, except that .02 N sodium acetate buffer was used.

In all the tissues, the digests were adjusted to pH 6-7, steamed and rehomogenized. These preparations were used for the assays of vitamin B_C and pantothenic acid; for biotin determinations an aliquot was autoclaved at 120°C for 2 hours in 6 N sulfuric acid and neutralized; for nicotinic acid an aliquot was autoclaved at 120°C for 20 minutes in 1 N sulfuric acid and neutralized; and for riboflavin and pyridoxine an aliquot was steamed at 100°C for one-half hour in 0.1 N hydrochloric acid, adjusted to pH 4.5 with sodium acetate, and filtered.

Vitamin Assays. The analyses of these tissues for their content of vitamin B_C (employing *L. casei* with the vitamin B_C reference standard of Parke, Davis & Co.), biotin, pantothenic acid, nicotinic acid, and riboflavin were performed by modifications of the

[1] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

[2] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[3] Johnson, M. J., *J. Biol. Chem.*, 1941, **137**, 575.

TABLE I.
Constituents of Media for Microbiological Assays.

Constituent	Amt./liter double-strength medium	
	Lactobacilli	*S. carlsbergensis*
Casein hydrolysate* (SMACO, 10% soln.)	100.00 ml	80.00 ml
Alkali treated peptone†	10.00 g	—
Yeast extract supplement†	2.00 "	—
Norit treated peptone‡	0.40 "	—
dl-Alanine‡	0.40 "	—
dl-Tryptophane	0.40 "	—
l-Asparagine	0.20 "	—
l-Cystine	0.20 "	—
Glucose	40.00 "	40.00 g
Sodium acetate (anhyd.)	24.00 "	—
Potassium citrate · H_2O§	1.00 "	10.00 "
Citric acid · H_2O	—	2.00 "
K_2HPO_4	1.00 "	0.60 "
KH_2PO_4	1.00 "	0.60 "
$MgSO_4 \cdot 7H_2O$	0.40 "	0.20 "
$FeSO_4 \cdot 7H_2O$	20.00 mg	10.00 mg
$MnSO_4 \cdot 4H_2O$	20.00 "	10.00 "
NaCl	20.00 "	10.00 "
Adenine sulfate	20.00 "	—
Guanine hydrochloride	20.00 "	—
Uracil	20.00 "	—
Xanthine	20.00 "	—
i-inositol	—	20.00 "
Nicotinic acid	2.00 "	—
Pyridoxine hydrochloride	2.00 "	—
Calcium pantothenate	1.00 "	2.00 "
Riboflavin	1.00 "	—
Thiamine hydrochloride	1.00 "	1.00 "
p-Aminobenzoic acid	0.10 "	—
Biotin	0.01 "	0.01 "
Adjust to pH	6.5-6.6	5.2-5.5

* Omitted in the riboflavin assay.[8]
† Added only in the riboflavin assay.[8]
‡ Added only in the vitamin B_c assay.[4]
§ Prevents precipitation of the mineral salts in the *Lactobacilli* assays.

existing microbiological methods.[4-8] The various media proposed for assays with the *Lactobacilli* have been incorporated into a single medium which, with the modifications indicated, serves for all of the *Lactobacilli* assays. The constituents of the medium are given in Table I, requiring only the omission of the vitamin under consideration for its assay. The assays employing the *Lactobacilli* were carried out in 5 ml quantities and inoculated by the convenient procedure suggested by Black and Arnold.[9] The *L. casei* were incubated at 37°C and the *L. arabinosus* at 30°C for 3 days and titrated electrometrically with 0.05 N NaOH. Pyridoxine was determined by the *S. carlsbergensis* method[10] employing the medium shown in Table I. The assays were run in 5 ml quantities contained in 50 ml Erlenmeyer flasks. The flasks were incubated for 18 hours at

4 Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, **157**, 303.

5 Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 95.

6 Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

7 Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

8 Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

9 Black, T. L., and Arnold, A., *Ind. and Eng. Chem., Anal. Ed.*, 1940, **12**, 344.

10 Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

30°C and growth was determined turbidimetrically in the Evelyn colorimeter.

Vitamin B_C assays on bone marrow samples extracted with ether at pH 4.5[11] indicated that fatty materials did not interfere with the assay. Recovery experiments on bone marrow for vitamin B_C and on liver for vitamin B_C and pantothenic acid also showed that the assays were functioning satisfactorily. The vitamin B_C contents of some samples of liver and bone marrow were also determined by the *Streptococcus fecalis R* method.[12] These values were 10-20% lower than those obtained by the *L. casei* method. Assays on the enzymes used for digestion of the tissues showed that there were insufficient quantities of the vitamins present to necessitate any correction in the values obtained.

The vitamin content of the livers and kidneys was expressed in amount per gram of dry tissue; while that of the bone marrow was expressed on the basis of amount per gram of nitrogen. We chose to express our vitamin concentrations per gram of nitrogen in the case of bone marrow since the constituents of this tissue are frequently expressed on the basis of nitrogen or phosphorus and because this procedure is experimentally convenient for bone marrow.

Experimental. Each of the experiments presented in this paper (Series I and Series II) consisted of 3 groups of animals: a *mustard-treated* group (fed *ad libitum*) which received topically 8 mg of mustard gas per kg of body weight and 2 *control* groups, one of which was fed *ad libitum* while the food allowance of the other was restricted to varying degrees as described below. Mustard gas was not administered to the control groups. The daily food consumption of each animal was recorded. The average weight of the animals at the beginning of the experimental period was 50 g in Series I and 46 g in Series II.

Series I. In the *mustard-treated* group, analyses were performed upon the tissues of 8 animals which died 3-7 days after the application of mustard. The average daily food consumption of these animals was 3.4 g and the average daily loss in weight was 3.1 g.

The food intake of each rat in the *control-restricted* group (8 rats) was limited to that consumed during the previous 24 hours by its paired member in the mustard group. Each animal was sacrificed 24 hours after the death of its paired-fed mate. The average daily loss of weight in this group was 1.3 g with considerable variation among animals in the total loss of weight over the 3-7-day period. The average daily food consumption of the *control-ad libitum* group (8 rats) was 8.5 g and the average daily weight gain was 3.2 g. The animals were sacrificed on the fifth and sixth days of the experiment.

In general, the concentrations of vitamins in tissues from the mustard-treated and food-restricted groups were similar and differed from those of the groups fed *ad libitum*. These changes were particularly evident in the liver, where marked increases were noted. The changes observed in this series were similar to those tabulated for Series II (Table III). Of significance for the purposes of this report is the fact that the deviations from the normal in the partially-starved group could be correlated with the degree of weight loss of the animals. This was particularly true for the concentrations of vitamin B_C which increased in the liver and decreased in bone marrow. As mentioned previously, the weight changes in the members of this group were variable and could be accounted for by the variations in the food intakes of their partners in the mustardized group. It was also apparent that the utilization of food by the rats poisoned with mustard was impaired to varying degrees in individual members of this group. Thus, the weight losses in these animals were uniform, in spite of the differences in food intake. These observations led to the conclusion that the vitamin changes in the mustard-treated animals were secondary to inanition and that the extent of the changes in the paired-fed rats was conditioned by the weight loss of the animals. Accordingly, in Series II, the food allowance of one control group was

[11] Bauernfeind, J. C., Sotier, A. L., and Boruff, C. S., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 666.

[12] Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

TABLE I.
Constituents of Media for Microbiological Assays.

Constituent	Amt./liter double-strength medium	
	Lactobacilli	*S. carlsbergensis*
Casein hydrolysate* (SMACO, 10% soln.)	100.00 ml	80.00 ml
Alkali treated peptone†	10.00 g	—
Yeast extract supplement†	2.00 "	—
Norit treated peptone‡	0.40 "	—
dl-Alanine‡	0.40 "	—
dl-Tryptophane	0.40 "	—
l-Asparagine	0.20 "	—
l-Cystine	0.20 "	—
Glucose	40.00 "	40.00 g
Sodium acetate (anhyd.)	24.00 "	—
Potassium citrate · H_2O§	1.00 "	10.00 "
Citric acid · H_2O	—	2.00 "
K_2HPO_4	1.00 "	0.60 "
KH_2PO_4	1.00 "	0.60 "
$MgSO_4 \cdot 7H_2O$	0.40 "	0.20 "
$FeSO_4 \cdot 7H_2O$	20.00 mg	10.00 mg
$MnSO_4 \cdot 4H_2O$	20.00 "	10.00 "
NaCl	20.00 "	10.00 "
Adenine sulfate	20.00 "	—
Guanine hydrochloride	20.00 "	—
Uracil	20.00 "	—
Xanthine	20.00 "	—
i-inositol	—	20.00 "
Nicotinic acid	2.00 "	—
Pyridoxine hydrochloride	2.00 "	—
Calcium pantothenate	1.00 "	2.00 "
Riboflavin	1.00 "	—
Thiamine hydrochloride	1.00 "	1.00 "
p-Aminobenzoic acid	0.10 "	—
Biotin	0.01 "	0.01 "
Adjust to pH	6.5-6.6	5.2-5.5

* Omitted in the riboflavin assay.[8]
† Added only in the riboflavin assay.[8]
‡ Added only in the vitamin B_c assay.[4]
§ Prevents precipitation of the mineral salts in the *Lactobacilli* assays.

existing microbiological methods.[4-8] The various media proposed for assays with the *Lactobacilli* have been incorporated into a single medium which, with the modifications indicated, serves for all of the *Lactobacilli* assays. The constituents of the medium are given in Table I, requiring only the omission of the vitamin under consideration for its assay. The assays employing the *Lactobacilli* were carried out in 5 ml quantities and inoculated by the convenient procedure suggested by Black and Arnold.[9] The *L. casei* were incubated at 37°C and the *L. arabinosus* at 30°C for 3 days and titrated electrometrically with 0.05 N NaOH. Pyridoxine was determined by the *S. carlsbergensis* method[10] employing the medium shown in Table I. The assays were run in 5 ml quantities contained in 50 ml Erlenmeyer flasks. The flasks were incubated for 18 hours at

4 Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1945, **157**, 303.

5 Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 95.

6 Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

7 Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **1941**, **139**, 675.

8 Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

9 Black, T. L., and Arnold, A., *Ind. and Eng. Chem., Anal. Ed.*, 1940, **12**, 344.

10 Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

Neither mustard treatment nor starvation produced any change in the percentage moisture content of the livers or kidneys.

Discussion. The foregoing data (Table III) demonstrate that severe starvation produces a marked rise in the content per gram of tissue of several of the B vitamins in the liver and that these changes also occur in the kidney, though to a lesser extent. The only notable change in the bone marrow is the fall in the vitamin B_C content. Although the riboflavin and pyridoxine values in the bone marrow show a trend in the opposite direction, it must be pointed out that the values in the starved and normal groups overlap to a great extent. Increases in the succinoxidase[13] and nucleoprotein[14] contents of liver during starvation have been reported.

It is also apparent (Table III) that the concentrations of the vitamins in the starved group are very similar to those in the mustard-treated group. It appears, therefore, that the changes in the vitamin content induced by mustard treatment are secondary to the concomitant inanition. The effect of mustard upon the vitamin B_C content of the bone marrow, however, appears to be specific. In this case the decrease in the vitamin due to the mustard treatment cannot be accounted for solely by inanition. This observation is of interest in view of the well-known deleterious effect of mustard upon the leukopoietic activity of the bone marrow. The prominent role of vitamin B_C in leukopoiesis suggests that the action of mustard upon the bone marrow is exerted through its effect upon the metabolism of vitamin B_C.

Table II shows that both mustard treatment and starvation cause a considerable decrease in liver size, both on an absolute basis and expressed as percentage of body weight. Under such circumstances it becomes necessary to reassess the significance of values based upon a unit weight of dry tissue. Two alternate methods of calculation present themselves: (a) determination of the total content of the constituent in the liver; or (b) calculation of the liver content per 100 g of body weight (percentage basis). Various investigators have elected to calculate the amounts of the following constituents in terms of one of these methods: vitamin,[15] enzyme,[13,16,17] nucleoprotein and phospholipid.[18]

Comparisons based upon the total amount of the constituent in the liver would appear to be valid only when the weights of the various groups of animals are the same. On the other hand, the expression of values in terms of the liver constituent per 100 g of body weight compensates for slight differences in body weight. Since the ratio of liver weight to body weight in normal rats varies during growth, such a calculation when applied to growing rats should be employed only when the ages of the different groups of animals under comparison are similar.

We believe that, under the conditions of the present experiment, calculations based upon the amount of liver constituent per 100 g of body weight furnish the most reliable basis for evaluating the significance of our data. Such calculations made from data for the liver given in Tables II and III (using amount of vitamin per unit of dry weight since the percentage dry weight is the same in the 3 groups) demonstrate that both starvation and mustard treatment cause increases ranging from 15 to 132% in the vitamin B_C, pantothenic acid, biotin and pyridoxine contents. No changes in the riboflavin and nicotinic acid values are observed. It is of interest that in the kidney, where the vitamin values are expressed per unit of dry weight of tissue, riboflavin and nicotinic acid are also the only 2 vitamins whose contents are not affected by starvation or mustard.

It is evident that the effects of inanition upon the contents of various liver constituents must be considered in cases where severe starvation for a short period of time

[13] Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 297.

[14] Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, **38**, 379.

[15] Sure, B., *J. Biol. Chem.*, 1945, **157**, 543.

[16] Lightbody, H. D., and Kleinman, A., *J. Biol. Chem.*, 1939, **129**, 71.

[17] Schultze, M. O., *J. Biol. Chem.*, 1939, **129**, 729.

[18] Kosterlitz, H. W., and Cramb, I. D., *J. Physiol.*, 1943, **102**, 18P.

TABLE II.
Changes in Liver Size Resulting from Mustard Treatment or Severe Starvation for 4 Days (Series II).*

Group	Starting wt (g)	Final wt (g)	Wt of liver (g)	% liver†
Control—*ad lib.* (10 rats)	46 (40-51)	54 (45-59)	3.10 (2.35-4.58)	5.74 (5.06-6.18)
Control—restricted (10 rats)	46 (39-50)	35 (27-39)	1.22 (0.69-1.67)	3.48 (2.56-4.00)
Mustard-treated (10 rats)	44 (40-46)	34 (31-36)	1.30 (1.03-1.60)	3.82 (2.86-4.56)

* Averages are given with the range of values in parentheses.
† Calculated from the final body weight.

TABLE III.
Effects of Mustard Treatment and of Severe Starvation upon the Concentrations of Vitamins of Rat Tissues (Series II).*

Group	Vitamin B_c	Pantothenic Acid	Biotin	Riboflavin	Pyridoxine	Nicotinic Acid
			Liver.			
Control—*ad lib.*	7.02 (5.50-8.30)	225 (151-304)	2.31 (1.50-3.82)	88.3 (62.4-116)	16.2 (10.4-24.8)	377 (334-426)
" —Restricted	19.7 (10.9-30.3)	428 (378-515)	5.01 (3.70-6.62)	134. (110-164)	42.6 (35.8-53.4)	554 (513-640)
Mustard-treated	24.3 (14.7-33.6)	433 (326-540)	4.71 (3.70-5.98)	131. (106-147)	39.5 (34.2-46.5)	546 (505-589)
			Kidney.			
Control—*ad lib.*	11.1 (7.78-13.6)	158 (146-168)	3.17 (2.28-3.76)	112. (104-124)	15.7 (11.4-22.0)	300 (280-320)
" —Restricted	14.4 (7.20-23.9)	260 (218-317)	4.94 (3.98-5.90)	111. (87.0-146)	25.4 (18.7-32.0)	310 (283-338)
Mustard-treated	16.2 (9.30-27.8)	293 (243-356)	5.04 (4.36-6.62)	104. (91.0-117)	23.3 (18.8-27.9)	306 (290-335)
			Bone Marrow.			
Control—*ad lib.*	26.1 (22.4-30.4)	360 (322-451)	1.56 (1.05-2.18)	262. (216-296)	36.2 (16.7-67.6)	1033 (815-1325)
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Mustard-treated	8.8 (4.9-14.6)	381 (285-500)	1.32 (0.54-1.82)	263. (171-398)	42.5 (32.4-50.7)	1220 (940-1570)

* The liver and kidney values are expressed as micrograms of vitamin per gram of dry weight; the bone marrow values are expressed as micrograms of vitamin per gram of nitrogen. Values given represent the averages of determinations on 10 samples, with the exception of vitamin B_c in bone marrow, in which case 20 samples were analyzed. Ranges are given in parentheses.

limited to amounts which kept the weight losses on a parity with those of the mustardized rats.

Series II. In the mustard-treated group, analyses were made of the tissues of animals which died on the fifth day after mustard gas application. The animals in the *restricted* group were each given 2 g of control ration daily for 3 days and no food on the fourth day. The following day the animals in this group, as well as those in the control group fed *ad libitum* were sacrificed. This degree of food restriction resulted in weight losses equal to those of the mustardized rats (Table II). This experiment was repeated exactly in order to obtain sufficient bone marrow to complete the vitamin analyses. The vitamin contents of the liver, kidney, and bone marrow from 10 animals of each group were determined, with the exception of the bone marrow vitamin B_C in which case 20 tissues from each group were analyzed.

Weight changes in the 3 groups, along with the total liver weight and the percentage of liver in the rats (based on final body weight) are given in Table II for purposes of calculations to be discussed. The vitamin contents of the tissues are given in Table III.

Neither mustard treatment nor starvation produced any change in the percentage moisture content of the livers or kidneys.

Discussion. The foregoing data (Table III) demonstrate that severe starvation produces a marked rise in the content per gram of tissue of several of the B vitamins in the liver and that these changes also occur in the kidney, though to a lesser extent. The only notable change in the bone marrow is the fall in the vitamin B_C content. Although the riboflavin and pyridoxine values in the bone marrow show a trend in the opposite direction, it must be pointed out that the values in the starved and normal groups overlap to a great extent. Increases in the succinoxidase[13] and nucleoprotein[14] contents of liver during starvation have been reported.

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We believe that, under the conditions of the present experiment, calculations based upon the amount of liver constituent per 100 g of body weight furnish the most reliable basis for evaluating the significance of our data. Such calculations made from data for the liver given in Tables II and III (using amount of vitamin per unit of dry weight since the percentage dry weight is the same in the 3 groups) demonstrate that both starvation and mustard treatment cause increases ranging from 15 to 132% in the vitamin B_C, pantothenic acid, biotin and pyridoxine contents. No changes in the riboflavin and nicotinic acid values are observed. It is of interest that in the kidney, where the vitamin values are expressed per unit of dry weight of tissue, riboflavin and nicotinic acid are also the only 2 vitamins whose contents are not affected by starvation or mustard.

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Weight changes in the 3 groups, along with the total liver weight and the percentage of liver in the rats (based on final body weight) are given in Table II for purposes of calculations to be discussed. The vitamin contents of the tissues are given in Table III.

TABLE I.
Toxicity of Amino Acids as Influenced by Riboflavin Deficiency.
10 rats to each set.

Diet	% dead Months 0	1	2	Avg wt, g Months 0	1	2
5% Tyrosine	0	0	0	47	84	162
" " Riboflavin def.	0	70	100	46	60	70
10% Histidine	0	30	40	46	68	82
" " Riboflavin def.	0	80	100	42	63	—
5% Cystine	0	0	20	51	102	173
" " Riboflavin def.	0	60	80	48	78	64
20% Glycine	0	20	40	50	74	98
" " Riboflavin def.	0	60	100	53	46	—
10% Glutamic acid	0	20	40	48	115	198
" " " Riboflavin def.	0	20	20	47	78	84
20% *dl*-tryptophane	0	20	40	42	75	63
" " Riboflavin def.	0	20	80	49	41	32
Riboflavin def.	0	0	10	41	78	89

acids occurred without exception in the 6 instances reported. This is established both by the increased death rate and by the decreased growth responses seen in the riboflavin-deficient animals. Tyrosine (5%), histidine (10%), and glycine (20%) were the most toxic amino acids in the riboflavin-deficient animals; whereas histidine (10%), glycine (20%), glutamic acid (10%) and tryptophan (20%) were the most toxic in the rats receiving riboflavin.

Discussion. The toxicity of tyrosine,[3,5] histidine,[2,6] glycine,[7] tryptophan,[5] cystine,[7] lysine,[5] serine,[8] methionine,[9] leucine,[6] hydroxyproline,[6] aspartic acid[6] has been reported. Glutamic acid is now added to this list. The results in this paper indicate increased toxicities for glycine, tryptophan, glutamic acid, cystine, histidine and tyrosine in the presence of a riboflavin deficiency. Balance is the deciding factor but there is specificity in these balance relationships as shown by the increased toxicity of tyrosine in a riboflavin deficiency[1] with no similar result in thiamin, pantothenic acid, folic acid, pyridoxine and vitamin A and D deficiencies. It is reflected in the ability of certain amino acids to counteract increased toxicities while others do not. As an example of this cystine decreased the toxicity of tyrosine,[1] while tryptophan and glutamic acid have no effect.

The explanation offered for consideration in the specific action of riboflavin deficiency in increased amino acid toxicities is that *l*-amino acid oxidase probably is a riboflavin-containing enzyme.[10]

Summary. *l*-Cystine, *dl*-tryptophan, *l*-tyrosine, *l*-histidine, glycine, and *d*-glutamic acid are more toxic in the riboflavin-deficient rat than in the rat receiving this vitamin.

[5] Sullivan, M. X., Hess, W. C., and Sebrell, W. H., *U. S. Pub. Health Rep.*, 1932, **47**, 75.

[6] Graham, C. E., Hier, S. W., and Klein, David, *Abstracts 110th Meeting A. C. S.*, 1946, 7K.

[7] Curtis, A. C., and Newburgh, L. H., *Arch. Int. Med.*, 1924, **29**, 828.

[8] Fishman, W. H., and Artom, C., *J. Biol. Chem.*, 1942, **145**, 345.

[9] Earle, D. P., Smull, K., and Victor, J., *J. Exp. Med.*, 1942, **76**, 317.

[10] Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, 1944, **152**, 119.

results from a given set of experimental conditions. This factor is of particular significance when one is dealing with pharmacological agents which often produce such a state of severe starvation.

Summary. 1. The vitamin B_C, pantothenic acid, biotin, riboflavin, pyridoxine, and nicotinic acid contents of the livers, kidneys, and bone marrows have been determined in rats: (a) fed *ad libitum;* (b) treated with mustard gas; and (c) whose food intake was restricted. 2. When expressed on the basis of unit weight of tissue, increases of 50 to 180% were noted in the vitamin concentrations of livers from the food-restricted group. Less marked increases occurred in the kidney, while a decrease of 35% in the vitamin B_C content of the bone marrow based on nitrogen content was observed. 3. With the exception of the vitamin B_C content of the bone marrow, the changes observed in the partially-starved group were also found in the mustard group. 4. Calculations based upon the vitamin content of the liver per 100 g of body weight indicate that both starvation and treatment with mustard gas cause increases ranging from 15 to 130% in the vitamin B_C, pantothenic acid, biotin, and pyridoxine contents of the liver. No significant changes occur in the riboflavin or nicotinic acid contents.

We are indebted to the Parke, Davis and Company for the vitamin B_c reference standard; and to Merck and Company for the synthetic vitamins. This work was done under a contract from the Medical Division of the Chemical Warfare Service.

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Toxicity of Amino Acids as Influenced by Riboflavin Deficiency.

GUSTAV J. MARTIN.

From the Research Department, The National Drug Company, Philadelphia, Pa.

The therapeutic application of protein hydrolysates has become common and as a result it grows more and more important to understand interrelationships between amino acids, vitamins, minerals, etc. In the course of experiments involving toxicity studies of histidine[1] and tyrosine,[2] it became probable that riboflavin deficiency was a key to increased amino acid toxicities. To establish this point, histidine and tyrosine toxicities were rechecked and cystine, glycine, glutamic acid and tryptophane were investigated.

Experimental. The various amino acids were incorporated into a synthetic diet[3] at varying levels from 5 to 20% depending upon the relative toxicity of the amino acid under consideration. The amino acid replaced an equivalent amount of carbohydrate in the basic diet. Glycine, *l*-tyrosine, *l*-histidine, *l*-cystine, *d*-glutamic acid and *dl*-tryptophan were used. The rats in all series showed crusted and bloody snouts, spectacled eyes, and diarrhea. The animals receiving glycine with or without superimposed riboflavin deficiency lost their hair; some became almost hairless. The rats were lethargic and appeared to lack muscular coordination. In those receiving glycine without a superimposed B_2 deficiency the hair condition corrected itself after a few weeks. Emaciation was common.

The symptomatology seen in these rats on toxic levels of an amino acid is also seen in riboflavin deficiency[4] but it should be recalled that these manifestations are nonspecific in nature.

Table I summarizes our results.

Accentuation of the toxicities of amino

1 Martin, G. J., in press.

2 Martin, G. J., in press.

3 Martin, G. J., *Arch. Biochem.*, 1943, **1**, 397.

4 McCollum, E. V., Orent-Keiles, E., and Day, H., *Newer Knowledge of Nutrition*, 1939, Macmillan, New York.

group being purebred Hampshires and half Lincoln X Rambouillet crossbreds. A group of 8 ewes was spayed in September, 1944, and maintained for a period of 90 days with a group of similar ewes serving as controls. At the end of this period, both groups were slaughtered and the pituitary glands removed for assay. All ewes in the spayed group had active corpora lutea in their ovaries at the time of the operation, and the control ewes had corpora lutea indicating recent ovulations at the time of slaughter. Thus, all ewes appeared to be in a reproductive condition normal for the breeding season.

A group of ewes from the same band was bred in the fall of 1944 and showed that they were reproductively normal by producing lambs during February and March of 1945. The lambs were weaned April 1, 1945 and on April 13 a group of 8 ewes was spayed. They too were maintained with a group of similar ewes which served as controls. All ewes were slaughtered after a period of 90 days and the pituitary glands removed for assay. At the time of spaying, all the ovaries removed were inactive with no follicle larger than 5 mm in diameter being observed. During the period from spaying until slaughter, an active painted teaser ram was kept with the control ewes and none showed an estrual period. Thus, all ewes were anestrual in a manner typical of the season. There was some ovarian activity since, at autopsy, one of the control ewes was found to have a fresh corpus luteum and 4 others had follicles as large as 8 mm in diameter. Three of 15 other ewes from the same band, slaughtered the same day, were found to have corpora lutea.

The pituitary glands were removed within 30 minutes after slaughter and immediately placed in an excess of acetone.† After being taken to the laboratory, usually a matter of from 4 to 5 hours, as much of the adhering connective tissue as possible was removed, the glands cut into small cubes, and the acetone changed. Three additional changes of acetone were made at intervals of 24 hours, after which drying was completed in desiccators containing calcium chloride.

After drying, the individual glands were ground with a mortar and pestle until fine enough to pass through an 80-mesh (to the inch) screen.

Assays of individual pituitary glands were carried out with rats and chicks. In the rat assays, the increase in ovarian weight of 21-day-old females was used as an endpoint. A total dosage of 20 mg of pituitary powder plus 4 mg of recrystallized hemin as an augmenter was suspended in 4½ cc of distilled water containing enough 1% NaOH to dissolve the hemin and injected subcutaneously in 9 equal injections over a period of 4½ days. Autopsies were performed 14 to 16 hours after the last injection. In the chick assays, the increase in testis weight of day-old White Leghorn cockerels was used as an endpoint. A total dosage of 10 mg of pituitary powder was suspended in 2½ cc of distilled water and injected subcutaneously in equal injections once per day over a 5-day period. Autopsies were performed 24 hours after the last injection.

The plan was to assay each gland on 2 rats and 5 chicks, but due to some death losses among the assay animals during the injection period, a few mistakes in sexing of the chicks, and a shortage of powder in 2 cases, data were actually available on a smaller number in some cases.

Results and Discussion. The results show that with both rats (Table I) and chicks (Table II) as assay animals the pituitary glands of spayed ewes of both breeds produced much greater responses than did the glands of the intact control ewes of the same breed. As indicated in Table III, these differences were highly significant. The differences between seasons were not significant in either breed in either spayed or control groups. There was no significant overall difference between seasons and the season-spaying interaction did not approach significance. Thus, the increase in gonadotrophic potency of the ewe pituitary glands after spaying was apparently of about the

† The cooperation of Armour and Co., Spokane, Washington, in making possible the collection of the glands in their plant is gratefully acknowledged.

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Gonadotrophic Potency of Ewe Pituitary Glands as Affected by Spaying, Season, and Breed.*

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The breeding season in ewes of most breeds is limited to the fall and early winter months.[1-4] During the remainder of the year, the ewe is anestrual although the ovaries often show a certain amount of activity, especially during the last few weeks before the breeding season begins.[1]

A lowered activity on the part of the pituitary gland has been postulated as a possible reason for anestrus in the ewe since injections of gonadotrophic hormones from pregnant mare serum or pituitary glands produce ovulation in a high percentage of anestrual ewes.[1,5] The great variability in response to injections of this kind,[6,7] however, raises a question as to whether other physiological factors may be involved.

The gonadotrophic hormone content of the pituitary gland of the male 13-lined ground squirrel increases greatly during the breeding season,[8] suggesting that at least in this species hormone content of the gland is correlated with secretory rate and the activity of the gonads. The gonadotrophic hormone content of the pituitary gland of the male cottontail rabbit increases during the breeding season, but that of the female does not.[9] The gonadotrophic hormone content of ewe pituitary glands remains about the same during the breeding season and during anestrus,[10] suggesting that a lack of pituitary gland activity may not be wholly responsible for anestrus in the ewe.

The time of the breeding season is evidently dependent upon environmental factors since it shifts when ewes are taken to the southern hemisphere.[5] The success which has been achieved in changing the breeding season by modifying the amount of light received is further evidence in this regard.[11] The physiological mechanism through which environmental factors operate to control sexual activity in the ewe has never been studied.

The hormonic secretions of active gonads normally lower the gonadotrophic hormone content of the pituitary gland and a pronounced rise occurs after gonadectomy.[12] Working upon the postulate that the pituitary gland of the anestrual ewe would indicate its potential activity by its ability to produce and store gonadotrophic hormones after spaying, the experiment reported here was performed in an effort to elucidate some of the factors responsible for the anestrual condition.

Materials and Methods. The experimental animals were 7-year-old ewes, half of each

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These data show that the pituitary gland of the anestrual ewe is comparable to that of the breeding-season ewe in having the ability to store gonadotrophic hormones in increased amounts following spaying. Thus the anestrual ewe has the ability to produce appreciable amounts of these hormones, and the suggestion is strong that this ability may be as great as in the breeding-season ewe. This latter point cannot be considered as proven, however, since the amount of hormone stored in the gland may reach an equilibrium in less than 90 days. The data provide no information on the rate of increase.

In view of the apparent ability of the anestrual ewe to produce gonadotrophic hormones, the current theory that anestrus is due to a lowered activity on the part of the pituitary gland may have to be modified. Quite possibly the immediate cause of the anestrual season is some changed physiological condition outside the pituitary gland which influences the ability of the ovary to respond to gonadotrophic hormones. Any attempt to discuss possible factors at this time would be mere speculation.

Summary. The gonadotrophic potency of pituitary glands from crossbred Lincoln × Rambouillet ewes was significantly higher than that of pituitaries from purebred Hampshire ewes. The gonadotrophic potency of glands from both breeds increased markedly during a 90-day period following spaying whether during the breeding season or during anestrus.

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Utilization of Glucose and Acetone Bodies by Gastrointestinal Tract in Fasting Normal and Diabetic Dogs.

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Although the method of arterio-venous differences has often been used to study the metabolism of muscle[1-5] and of brain[6,7] in both normal and diabetic animals and men, this procedure has apparently seldom been applied to the gastrointestinal tract. It is now generally recognized that the RQ of the brain is always close to unity, and that it continues to remove glucose from the blood and utilize this sugar as its chief, if not its only fuel, under a great variety of conditions including fasting and diabetes. In skeletal muscle, however, it has been found by all who have studied A-V differences in severe diabetes that either no glucose is removed or the amount removed is greatly reduced even after the administration of this sugar. Only Cavett and Seljeskog[4] report that as much glucose is removed in diabetic patients as in normal controls, but it seems improbable that they studied severe diabetes for only one of their 8 patients had a blood sugar level above 180 mg/ml. Others have also reported nearly normal values in mild diabetes,[1,3] but absent or reduced A-V differences in severely diabetic patients.

London[8] found that the portal blood of normal dogs contained 6 to 9 mg/100 ml

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TABLE I.
Ovarian Responses of Immature Rats to Injections of 20 mg of Pituitary Powder and 4 mg of Recrystallized Hemin.

Season in which donor ewes slaughtered	Type of donor ewes: Intact — No. ewes	No. rats	Avg ovarian wt. mg	Spayed — No. ewes	No. rats	Avg ovarian wt. mg
Breeding season:						
Hampshire	4	8	15.0 ± 1.3*	4	7	34.1 ± 17.3
Crossbred	4	7	27.9 ± 8.2	4	8	134.5 ± 45.0
Anestrus:						
Hampshire	4	8	13.4 ± 1.8	4	8	36.0 ± 8.2
Crossbred	4	8	20.0 ± 5.7	4	8	83.7 ± 19.8

* Standard errors based on between ewe mean squares.

TABLE II.
Testicular Responses of Immature Chicks to Injections of 10 mg of Pituitary Powder.

Season in which donor ewes slaughtered	Type of donor ewes: Intact — No. ewes	No. chicks	Avg testis wt. mg	Spayed — No. ewes	No. chicks	Avg testis wt. mg
Breeding season:						
Hampshire	4	17	17.4 ± 2.4*	4	17	19.5 ± 2.8
Crossbred	4	16	19.1 ± 3.0	4	17	25.2 ± 4.6
Anestrus:						
Hampshire	4	12	14.0 ± 2.9	4	13	22.1 ± 2.8
Crossbred	4	13	18.0 ± 3.7	4	14	22.1 ± 1.6

* Standard errors based on between ewe mean squares.

TABLE III.
Significance of Various Factors Influencing the Gonadotrophic Potency of Ewe Pituitary Glands as Determined by Analysis of Variance.

	Type of assay: 21-day-old female rat	Day-old cockerel
Season	N.S.	N.S.
Breed	**	*
Spaying	**	**
Season × Breed	N.S.	N.S.
Season × Spaying	N.S.	N.S.
Breed × Spaying	N.S.	N.S.
Season × Breed × Spaying	N.S.	N.S.

Meanings of symbols are as follows:
N.S.—Not statistically significant.
*—Statistically significant $P < .05$.
**—Statistically highly significant $P < .01$.

same order during anestrus as during the breeding season.

The pituitary glands of the Lincoln × Rambouillet crossbred ewes were consistently more potent than those of Hampshire ewes, and as indicated in Table III, the differences were significant or highly significant as judged by the chick or rat assays, respectively.

The variances associated with the group means in the chick assay data were essentially homogeneous within the various groups and therefore well suited to the use of analysis of variance without transformation. The variances in the rat assay data were distinctly heterogeneous, and it was necessary to transform the ovarian weights to the logarithms of their logarithms before the mean squares seemed independent.‡

Qualitative as well as quantitative differences may exist between glands in their gonadotrophic hormone content. The degree of luteinization in the ovaries of the test rats was observed to be related to ovarian weight. Whether this represented a qualitative difference or merely a dosage effect is not known. Glands from spayed ewes in the 2 seasons were very comparable in their ability to produce luteinization.

‡ The interest and cooperation of Professor George W. Snedecor, Iowa State College, Ames, Iowa, in suggesting and making these transformations is gratefully acknowledged.

TABLE I.
Normal Dogs.
Arterio-portal Differences of Glucose, Lactic Acid, and Acetone Bodies.

	Mean A-P differences and standard errors		
	Glucose*	Lactic acid*	Acetone bodies*
Total No. of samples	57	38	37
Mean value, mg/100 ml	+1.8 ± 0.3	—0.1 ± 0.22	+0.38 ± 0.11
G-I removal: No. samples†	30	6	14
Range‡	+2 to +6	+0.6 to +5.4	+0.6 to +1.8
No change: No. samples†	23	20	21
Range‡	+1 to —1	+0.5 to —0.5	+0.5 to —0.5
G-I additions: No. samples†	4	13	2
Range‡	—2 to —5	—0.6 to —2.5	—0.6 to —0.9

* + indicates removal by gastrointestinal tract, — addition to blood.
† No. samples indicates the number of times that an amount of substance beyond the limits of error of the chemical method was removed or added, all others considered as "no change."
‡ "Range" indicates the range of value taken as indicating removal, no change, or addition, respectively.

TABLE II.
Diabetic Dogs.
Arterio-portal Differences of Glucose, Lactic Acid, and Acetone Bodies.

	Mean A-P differences and standard errors.		
	Glucose*	Lactic acid*	Acetone bodies*
Total No. of samples	33	33	33
Mean value, mg/100 ml	+3.2 ± 0.6	—0.37 ± 0.16	+1.8 ± 0.45
G-I removal: No. samples†	21	4	25
Range‡	+2 to +10	+0.6 to +2.5	+1.1 to +7.8
No change: No. samples†	8	15	5
Range‡	+1 to —1	+0.5 to —0.5	+1.0 to —1.0
G-I additions: No. samples†	4	14	3
Range‡	—2 to —3	—0.6 to —2.5	—1.1 to —5.3

*, †, ‡ as in Table I. Note that the range considered to be within the limits of error of the acetone body method is 1.0 mg/100 ml in diabetic dogs as compared with +0.5 in normal dogs; the higher range for the depancreatized animals is due to their higher blood acetone body level.

TABLE III.
Comparison of Arterial Blood Levels and Arterio-portal Differences in Dogs Fasted 3 and 5 Days.
(None of these changes is statistically significant.)

	Glucose		Lactic acid		Acetone bodies	
	Arterial blood conc.	A-P diff.	Arterial blood conc.	A-P diff.	Arterial blood conc.	A.P. diff.
3 day fasted	69	1.9	8.3	+0.2	2.9	0.23
5 " "	72	1.3	8.5	—0.35	5.6	0.53

the diabetic dogs were: glucose, —336, lactic acid, —13.4, and acetone bodies —67.9. The blood sugar and lactate levels showed a tendency to increase somewhat on the fifth, sixth, and seventh days after withdrawal of food and insulin, although no statistically valid correlation can be made. The acetone body concentration averaged 37.6 on the third day and 86.3 on the fourth or later days, the highest value attained being

less glucose than the arterial, and that lactic acid might be either removed or added. Cherry and Crandall,[9] in dogs fasted 18 hours, observed a difference of 2.9 mg of glucose and no significant change in lactic acid. In amytalized dogs fasted 28 hours, Wierzuchowski and Fiszel[10] report the removal of 1.9 mg of glucose and the addition of 3.1 mg of lactic acid.

In the course of determinations on hepatic glucose output and blood flow in normal[11] and diabetic[12] dogs, an opportunity was presented to obtain data on the retention or output of glucose, lactic acid and acetone bodies by the gastrointestinal tract.

Methods. London cannulae were applied to the portal (and hepatic) veins of normal dogs by the usual method. Other animals were similarly angiostomized and the pancreas completely removed at the second stage of the operation. Survival of the angiostomized-depancreatized dogs was promoted by postoperative treatment with sulfathiazole. Both normal and depancreatized animals were trained until they were not disturbed by the procedure of withdrawing blood. The diabetic dogs were kept on a diet of beef heart, raw pancreas, and milk with sufficient insulin (regular and protamine separately injected once daily) to reduce the urinary glucose loss to 1 or 2 g per day; these animals were not used until they had been stable on this regime for at least 2 weeks. The normal dogs were fasted 3 to 5 days. Blood samples were then taken from the femoral artery and portal vein at intervals of 1 or 2 hours until 3 or 4 pairs of samples had been removed. Similar pairs of samples were taken from the depancreatized dogs 3 to 7 days after withdrawal of food and insulin. All blood samples were analyzed for glucose by the method of Shaffer and Somogyi,[13] lactic acid by the method of Barker and Summerson,[14] and acetone bodies by the method of Crandall.[15]

Results. In Tables I and II we present the data on arterio-portal differences in normal dogs and in depancreatized dogs that have been without food and insulin for 3 or more days. Since blood sugar levels and urine glucose loss in the depancreatized dogs became stabilized on the third day, we consider that the latter animals were completely diabetic at this time.

It will be noted that the gastrointestinal tract of the normal dog removes significant amounts of glucose and acetone bodies from the arterial blood, but has no consistent effect upon the concentration of lactic acid. In more than half of all pairs of samples, there was no appreciable effect on lactic acid concentration, and although lactic acid was removed more often than it was added, the behavior of the gastrointestinal tract toward this substance is unpredictable. These statements apply equally to animals fasted 3 and 5 days.

There are suggestive differences with respect to glucose and acetone bodies between dogs fasted 3 days and those fasted 5 days as shown in Table III, although none of these differences is statistically significant.

The utilization of glucose, in terms of quantity per unit blood flow, is seen to be 1.6 mg/100 ml higher in the diabetic dogs than in normal animals. This is an increase of 90% over the normal value, but the difference only approaches statistical significance; $t = 2.1$, $P < 0.05$. In the diabetic dog there is also an appreciable utilization of lactic acid, which is probably significant, and the utilization of acetone bodies is 1.4 mg/100 ml greater than in normal dogs. The "t" for the difference in acetone utilization between normal and diabetic dogs is 3.1, $P < 0.001$. The average blood levels in

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15662

Acetylation of Sulfanilamide as Influenced by the Thyroid.*

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The acetylation of sulfanilamide has proved to be a useful reaction for the determination of the acetylating capacity of the animal organism. The reaction has been shown to take place in the liver[1,2] in most animal species and to be irreversible.[3-5] An increase in the degree of acetylation was observed following the administration of various metabolites, such as acetate,[6-9] pyruvate,[7,10] lactate,[7] alcohol[6] and glucose.[7] The *in vitro* experiments of Klein and Harris[2] and of Lipmann[11] demonstrated an increased reactivity if acetate, pyruvate, lactate, acetoacetate, or acetoin were added to the reaction vessels. Lipmann, using pigeon liver as the most adaptable tissue for these studies, found the conjugation to take place not only in the slice but also in homogenates and even in extracts of the liver.

Inasmuch as the thyroid greatly affects metabolism and products of intermediary metabolism have been shown to influence the degree of acetylation of administered aromatic amines, a study of the effect of the thyroid on the *in vivo* acetylation of sulfanilamide was undertaken.

Methods and Results. The experiments here reported were carried out on female rats weighing approximately 200 g. The series consisted of untreated controls, rats which had received a diet consisting of 1% desiccated thyroid for 2 to 3 weeks, rats which had been thyroidectomized 2 to 3 months prior to the experiment, and rats which had received 0.1% thiouracil in their drinking water for 3 weeks. The animals were fasted overnight, injected with 5 ml of approximately 1% sulfanilamide solution, put in metabolism cages with free access to food and water for 24 hours, and the urine collected and analyzed according to the method of Bratton and Marshall.[12] The total amount of sulfanilamide excreted was approximately the same in all instances. The per cent of this amount which was acetylated varied with the experimental conditions; these data are recorded in Table I.

Statistical analyses[13] of the data were carried out by the standard procedure for small samples of determining the standard error of the difference between the 2 means. In the experiments using hyperthyroid rats, the difference between the means of the control and experimental groups was more than 3 times the standard error, the difference between the means of the controls and thiouracil-treated group was 2.5 and between the controls and throidectomized group it was 2.2. Taken in conjunction with the probability of the occurrence of the deviations derived from Fisher's "t" table, which is shown in Table I, the difference found in the hyperthyroid group appears to be definitely significant; the differences in the thyroid-

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210 mg/100 ml in one dog on the fourth day.

Discussion. From the data presented above we may conclude that the gastrointestinal tract of the fasting normal dog utilizes glucose and acetone bodies, but has no consistent effect on the concentration of lactic acid. It should be noted that removal of appreciable quantities of acetone bodies could not be expected to occur until the animal had been without food for at least 24 hours, for in the postabsorptive state these substances are not being manufactured by the liver and their concentration in the blood is insignificant.[16] We may also conclude that the gastrointestinal tract of the depancreatized dog from which food and insulin have been withheld for at least 3 days: (1) removes glucose possibly at a greater rate than the G-I tract of the normal animal, (2) adds lactic acid to the blood, and (3) removes acetone bodies at a more rapid rate than is true of the normal dog, the difference being in line with the higher blood acetone body concentration of this preparation.

Our present observations on normal dogs confirm the removal of glucose and inconsistent effect on lactic acid previously noted in normal unanesthetized dogs,[8,9] although the quantity of glucose taken up is less than reported by London.[8] The uptake of lactic acid reported by Wierzuschowski and Fiszel[10] may be attributed to their use of amytal anesthesia, since it has been shown that anesthetics may alter metabolic processes.[17] Utilization of acetone bodies by the normal gastrointestinal tract and the A-P differences in depancreatized dogs do not appear to have been reported previously. The structures drained by the portal vein may now be ranked with skeletal muscle in their ability to use acetone bodies, and brain remains the only tissue which has been shown not to remove them.[18]

Of particular interest is the continued utilization of glucose by the gastrointestinal tract in the diabetic dog. Assuming that the portal blood flow is 1.5 liters/kg/hour, our value would indicate the removal of 48 mg of glucose/kg/hour. Of this quantity some 11.6% is returned to the blood as lactic acid, leaving about 42 mg/kg/hour unaccounted for and presumably oxidized. This quantity would yield nearly 4 calories per kg per day. It has been shown that brain utilizes little, if any, other fuel than glucose in diabetes and accounts for about 10% of the total resting metabolism.[7] If then the utilization of glucose by the gastrointestinal tract accounts for another 8%, approximately 18% of the heat production of the resting diabetic dogs could be accounted for by the oxidation of glucose.

This inference, that 88% of the glucose removed by the gastrointestinal tract is oxidized, must of course be regarded as tentative, pending the demonstration of the actual fate of this sugar. However, it is difficult for us to conceive of any other explanation for its disappearance.

Summary. In the normal dog fasted 3 or 5 days, the organs drained by the portal vein remove significant amounts of glucose and acetone bodies, but have no consistent effect upon the blood lactic acid. In the depancreatized dog, 3 to 7 days after the withdrawal of food and insulin, these organs continue to remove statistically significant quantities of glucose and increase their removal of acetone bodies. Under these conditions they add lactic acid to the blood. If the glucose removed by the gastrointestinal tract of the depancreatized dog and not returned to the blood as lactate is oxidized, it will account for approximately 8% of the animal's resting metabolism.

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TABLE I.
In vitro Effect of Penicillin on the Coagulation Time of Normal and Hemophiliac Whole Blood.

Final conc. of penicillin (units) per 2 cc of blood	Coagulation time (min)	
	Normal	Hemophiliac
1000	6.5	33
250	6.5	32
25	6.5	33
5	8	30
2.5	8	30
1.25	8	32
0.75	7.5	32
0.5	7.5	32
0.25	7.5	32
0	7	30

Clot retraction normal in all tubes.

oughly the effects of penicillin on blood coagulation in both normal and hemophiliac subjects.

Methods and Material. Six normal and 6 hemophiliac subjects were studied: whole blood coagulation times by a modification[3] of the method of Lee and White at 37°C, prothrombin times by a modification[4] of the Quick Method[5] and clot retraction were determined repeatedly in all patients. Capillary bleeding times, platelet counts and plasma fibrinogen concentrations were determined in many of the experiments. Penicillin levels[6] were determined in all patients.

In vitro tests were performed to determine the effect of penicillin at various concentrations on the coagulation time of normal and hemophiliac blood. Two cc of whole blood was added to 0.1 cc of various 0.85% sodium chloride dilutions of penicillin and coagulation times measured.

Two normal and 2 hemophiliac subjects received oral penicillin in an initial dosage of 300,000 units, followed by 100,000 every 2 hours for 48 hours. Four normal and 4 hemophiliac patients received intramuscular sodium penicillin in single doses of 25,000, 50,000, 100,000 and 200,000 units each.

Results. In vitro: No effect was produced on the coagulation time of normal or hemophilic blood by the *in vitro* addition of various dilutions of penicillin. The results are shown in Table I.

In vivo: 1. Oral penicillin. No effect on the blood coagulation mechanism was observed after oral administration of penicillin in either normal or hemophiliac subjects. One typical case, a hemophiliac, is presented in Table II.

2. Intramuscular penicillin. Four normal and 4 hemophiliac patients received intramuscular penicillin in a single dose of 25,000, 50,000, 100,000 or 200,000 units without effect on coagulation time, clot retraction, prothrombin time, platelet count, bleeding time or fibrinogen concentration in spite of high plasma penicillin levels.

One normal and one hemophiliac subject, each of whom received 200,000 units of penicillin intramuscularly are presented in Tables III and IV.

Discussion. Penicillin is used with such frequency that any contraindication to its use should be thoroughly investigated. The suggestion that penicillin might alter the blood coagulation reaction to such an extent that intravascular coagulation could be produced or increased is important particularly in view of the frequency of penicillin ad-

TABLE II.
Effect of Oral Penicillin (100,000 Units Every 2 Hours) on the Blood Coagulation of a Hemophiliac Subject.

Time	Coagulation time, (min)	Clot retraction	Prothrombin time, (sec)	Fibrinogen, mg/100 cc	Penicillin level, units/cc
Before penicillin	150	normal	21	383	0
24 hr of penicillin	160	"	22	371	0.11
48 " " "	160	"	22	338	0.22

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TABLE I.
Acetylation of Sulfanilamide.

Exp. condition	% acetylated Exp.	% acetylated Control*	Diff.	P‡
Hyperthyroidism	30 ± 1.8 (11)†	39 ± 1.6 (11)	—23%	.01
Thyroidectomy	33 ± 2.2 (7)	39 ± 1.2 (6)	—15%	.05
Thiouracil treatment	44 ± 1.5 (11)	39 ± 1.4 (11)	—13%	.05

* For each experimental animal a control animal was run at the same time to minimize differences due to temperature, humidity, diet, etc.

† Figures represent mean values ± the standard error. Numbers in parentheses refer to number of rats per group.

‡ Probability of occurrence of deviation, obtain from Fisher "t" table.

ectomized- and thiouracil-treated groups just barely significant.

The results show that both hyperthroidism and thyroidectomy produced a decrease in the degree of acetylation of sulfanilamide, whereas thiouracil treatment caused an increase. It is difficult to interpret the variance in the effects of thyroidectomy and thiouracil. A possible explanation may be that thiouracil exerts other effects than those depressing the activity of the thyroid.

It may be noteworthy to compare these results with the changes observed in an entirely different experiment, in which the high energy containing phosphate compounds of the liver were determined under similar conditions;[14] hyperthyroidism was associated with a decrease in the specific activity of the labile phosphorus, thyroidectomy with no change in this value, and thiouracil treatment with an increased specific activity of the labile phosphorus.

Summary. The influence of the thyroid on the acetylation of sulfanilamide was studied. Hyperthyroidism and thyroidectomy caused a decrease in the amount of sulfanilamide acetylated; thiouracil treatment led to an increase.

[14] Greenberg, D. M., Fraenkel-Conrat, J., and Glendening, M. B., unpublished observations.

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Effect of Penicillin on Blood Coagulation.*

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Moldavsky, Hasselbrook and Caterno[1] have recently reported that both oral and intramuscular penicillin consistently shorten the coagulation time of normal blood and produce a nonretractile clot. These authors also observed decreased bleeding times in patients receiving penicillin. Hines and Kessler[2] demonstrated increased sensitivity to heparin in 2 of 10 cases receiving penicillin but found no changes in the platelet counts or prothrombin times.

The widespread use of penicillin makes this an important clinical problem. Therefore, it was decided to investigate more thor-

* The expenses of this investigation were defrayed in part by a gift to Harvard University from Smith, Kline and French Laboratories, of Philadelphia, and in part by a grant "in recognition of Dr. Francis W. Peabody's services" to the Foundation by the Ella Sachs Plotz Foundation.

[1] Moldavsky, L. F., Hasselbrook, W. B., and Caterno, C., *Science*, 1945, **102**, 38.

[2] Hines, L. E., and Kessler, D. L., *J. A. M. A.*, 1945, **128**, 794.

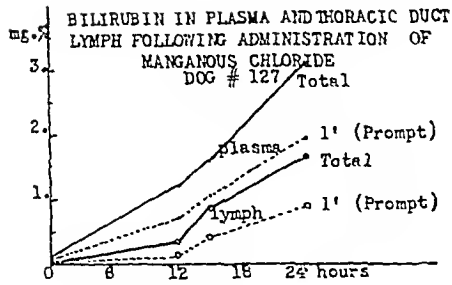

FIG. 1.

be toxic to the liver of rabbits, rats, and guinea pigs.

Material and Methods. Seven male dogs were used, ranging from 18 to 20 kg in weight. The anesthetic employed was pentobarbital sodium in an amount of 35 mg per kg of body weight. The thoracic duct lymph was secured in the following manner: In accordance with the Markowitz technic[6] the left subclavius vein was ligated, as were also the veins entering the external jugular just above its junction with the subclavius. Instead of ligating the innominate vein, as recommended by Markowitz, a string was placed around it just below the entrance of the thoracic duct. The left external jugular was then ligated and cut at a distance of 6-7 cm above the clavicle. A plastic cannula 3 cm in length* was introduced in the proximal cut end of the external jugular vein. A sample of thoracic duct lymph could then be secured by pulling the string upward, thus impeding the flow of lymph into the general circulation. At other times, the lymph flows through its natural channel, the string being loose and a small cork stoppering the cannula.

Four of the dogs were given 5 cc each of carbon tetrachloride, together with 5 cc of 95% ethyl alcohol by stomach tube immediately following thoracic duct cannulation and again after 24 hours. Each of the remaining 3 dogs were given 800 mg of $MnCl_2$ dissolved in 20 cc of physiological saline. Twenty-four hours later a second dose of 400 mg was injected into each of 3 dogs. These amounts were injected intramuscularly.

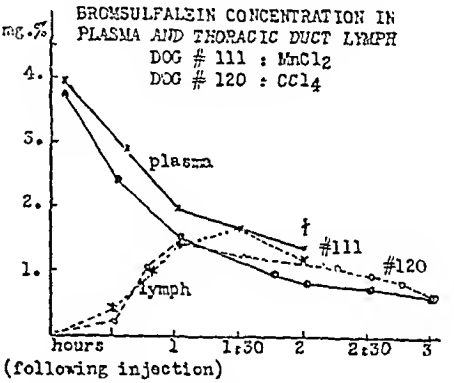

FIG. 3.

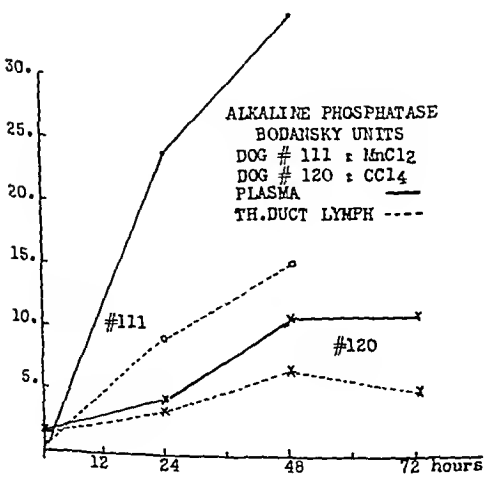

FIG. 2.

The laboratory methods used were the same as those described in a previous communication,[7] except for the alkaline phosphatase which was determined by the Bodansky method.[8]

Results. The bilirubin increased in both blood and lymph 12 hours following the first

[6] Markowitz, J., *Textbook of Experimental Surgery*, William Wood and Co., 1937.

* The author is indebted to Dr. J. H. Grindley, of the Mayo Clinic, Rochester, Minn., for suggesting the use of this type of cannula, and to the Irvington Varnish and Insulator Co., Irvington, N.J., for making it available.

[7] Gonzalez-Oddone, Miguel V., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 144.

[8] Bodansky, A., *J. Biol. Chem.*, 1937, **120**, 167.

TABLE III.
Effect on Blood Coagulation of 200,000 Units of Penicillin Intramuscularly in a Normal Subject.

Time (hr after injection)	Coagulation time (min)	Prothrombin time (sec)	Clot retraction	Bleeding time (min)	Fibrinogen, mg/100 cc	Penicillin, unit/cc
Initial	8½	22	normal	1	243	0
½	9	19	"	1	236	7.15
2	7½	21	"	1¼	232	0.45
3	7½	22	"	1¼	204	0.11
4	8	22	"	1	231	0.05

TABLE IV.
Effect on Blood Coagulation of 200,000 Units of Penicillin Intramuscularly in a Hemophiliac Subject.

Time (hr after injection)	Coagulation time (min)	Prothrombin time (sec)	Clot retraction	Bleeding time (min)	Platelet count, × 1000	Fibrinogen, mg/100 cc	Penicillin, unit/cc
Control	79	20	normal	1½	252	224	0
1	110	20	"	1¾	289	339	7.15
2	80	20	"	2¼	250	336	0.90
3	97	19	"	2¼	258	256	0.11
4	105	21	"	2	262	286	0.05

ministration to patients suffering from thrombophlebitis, coronary artery disease and bacterial endocarditis. We were unable to demonstrate any effect of penicillin on either the normal or hemophiliac blood coagulation reaction, and therefore do not feel that consideration of intravascular blood coagulation offers any contraindication to the clinical use of penicillin.

Conclusions. We were able to demonstrate any *in vitro* or *in vivo* effect of penicillin on the blood coagulation reaction. No significant changes were observed in coagulation time, clot retraction, prothrombin concentration, fibrinogen concentration, platelet count or bleeding time of normal or hemophiliac subjects.

We are indebted to Clare Wilcox for the penicillin determination.

15664 P

Studies of the Thoracic Duct Lymph in Experimental Liver Injury in Dogs.

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The purpose of the present communication is to report comparative studies of the bilirubin, alkaline phosphatase, cholesterol, bile acids and intravenously-administered bromsulfalein content of the blood and thoracic duct lymph of dogs acutely poisoned with carbon tetrachloride or manganous chloride. A number of investigators[1-3] have used carbon tetrachloride by stomach tube to produce liver injury. A marked increase in its toxicity can be obtained by simultaneous administration of ethyl alcohol.[4] Manganous chloride was shown by Findlay[5] to

1 Meyer, J. R., and Pessoa, B. S., *Am. J. Trop. Med.*, 1923, **3**, 177.

2 Lamson, P. D., and McLean, A. J., *J. Pharm. and Exp. Therap.*, 1923, **21**, 237.

3 Freeman, S., Chen, Y. P., and Ivy, A. C., *J. Biol. Chem.*, 1938, **124**, 79.

4 Lamson, D. P., Gardner, G. H., Gustafson, R. K., Maire, E. D., McLean, A. J., and Wells, H. S., *J. Pharm. and Exp. Therap.*, 1923, **22**, 215.

5 Findlay, G. M., *Brit. J. Exp. Path.*, 1924, **5**, 92.

tistically significant changes, although the electrocardiograms remained within clinically normal limits. In 20 cardiac patients, mostly definite or suspected coronary disease, we recorded the standard limb leads, 3 chest leads (CF_1, CF_2, CF_4) and the heart sounds before and at 30 and 60 minutes after a 1000-calorie meal. In 2 patients additional chest leads were taken. The location for the chest leads was marked on the chest by means of a colored pencil.

Out of 10 patients with some symptoms of angina pectoris but with normal electrocardiogram before the meal and without any signs of decompensation, the electrocardiogram became abnormal after the meal. The abnormality involved inversion of the T-wave in lead CF_2 or CF_4 or increase in the size of a Q-wave in lead III. These patients felt no discomfort after the meal.

In 5 patients with coronary disease and abnormal electrocardiogram before the meal, the electrocardiogram became more abnormal with inversion of the T-wave in leads 1, 2 or CF_4. Slight changes of the QRS-contour were also observed. In one patient, a transitory *pulsus trigreminus* appeared after the meal.

In 2 patients with abnormal electrocardiograms before the meal the chest leads appeared to be more normal after the meal; previously inverted T-waves in CF_2 and CF_4 became positive. Similar changes have been observed after exercise in coronary patients and cannot be interpreted as a sign of improvement.

In 2 patients with coronary disease and abnormal electrocardiograms, the meal produced violent discomfort, associated with a pronounced ST-depression, especially in lead II. The discomfort as well as the ST-depression was instantaneously removed by nitroglycerine. In normal subjects the ST-segment does not change after the meal. It seems probable that the ST-depression is due to a transitory coronary insufficiency, produced by an abnormal reflex coronary constriction. In only one patient with an abnormal ECG were the changes after the meal comparable to those observed in normal subjects.

The present series is only of an exploratory nature, and no attempt is made to correlate the effect of meals on the electrocardiogram to the nature of the existing pathological changes. However, the series appears to show that the effect of meals is of importance for the clinical interpretation of electrocardiograms and for the standardization of procedure.

15666

Radiosodium Tracer Studies in Congestive Heart Failure.*

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Extensive studies in this laboratory on the distribution and turnover of sodium in cardiovascular states with Na^{24} (14.8 hour ½ life) as a tracer led to the possible investigation of long-term phenomena associated with congestive heart failure and edema. For such studies a long life isotope is necessary. Radioactive sodium 22 (3 year ½ life) is listed in the isotope tables but has not been reported as used in biological studies. Through the cooperation of Professors M. D. Kamen and A. L. Hughes of the Washington University Cyclotron, an experimental supply was made available for investigation. The details of preparation, standardization, determination and safety will be described later. In view of the significant results as well as for the introduction of this technic in the

* Aided by grants from The Helis Institute for Medical Research, Mrs. E. J. Caire Fund for Cardiovascular Research, and The Life Insurance Medical Research Fund.

dose of $MnCl_2$ and 24 hours following the first dose of CCl_4. The main component of the blood and lymph bilirubin was the 1′ or prompt reacting type.[9,10] Fig. 1, from one of the $MnCl_2$ dogs is a typical example. The alkaline phosphatase increased as early as 12 hours following the first dose of $MnCl_2$ in both lymph and blood, and a definite increase was noted in all of the dogs 24 hours following the administration of either of the toxic substances. Fig. 2 compares the typical results from $MnCl_2$ and CCl_4 (one dog for each).

The total cholesterol changed very little in any of the experiments, either in blood or lymph, if anything showing a slight tendency to decrease. The same might be said for the cholesterol esters. The bile acid concentrations in blood and lymph showed no significant alterations in the experiments with either of the 2 toxic substances. The behavior of the intravenously-injected bromsulfalein was similar in all 6 of the dogs in which it was administered (3 $MnCl_2$ and 3 CCl_4 dogs). As noted in Fig. 3, the bromsulfalein was retained in the blood at relative high concentration, a delayed increase appearing in the thoracic duct lymph similar to that previously described for the 32-hour stage following common duct ligation.

Discussion. The present results differ in a number of respects from those previously described for experimental obstructive jaundice.[7] The increase of serum bilirubin in the lymph was not as early nor as marked, and the same was true for bromsulfalein, although, as noted above, there was a delayed increase in the lymph, similar to that seen 32 hours following common duct ligation. The behavior of the bile acids was quite different in that, in the present experiments, no increase was noted, while in the experimental obstructive jaundice, the bile acids rose rapidly and to a marked degree in the lymph. The alkaline phosphatase behaved quite similarly in the 2 groups of experiments. While no attempt will be made to explain these observations, the possibility is considered that the differences are due to a relatively greater hepatocellular and lesser cholangiolar injury in the present experiments than in the experimental obstructive jaundice.

Summary. A method is described that allows collection of thoracic duct lymph over a period of days, the lymph circulating through its natural channel during the intervals in which it is not being collected. Liver injury caused by either CCl_4 or $MnCl_2$ were qualitatively comparable in dogs, the changes being more marked with the latter. An elevation of the alkaline phosphatase activity and bilirubin content of both blood and thoracic duct lymph were noted. The main component of the bilirubin was the 1′ or prompt reacting type.[9,10] The behavior of the injected bromsulfalein was comparable to that of the 32-hour stage, following common bile duct ligation.[6] No significant changes were observed as regards the bile acids, total cholesterol or cholester esters.

9 Ducci, H., and Watson, C. J., *J. Lab. and Clin. Med.*, 1945, **30**, 293.

10 Watson, C. J., *Blood*, 1946, **1**, 99.

15665 P

Effect of Meals on the Electrocardiogram of Cardiac Patients.*

ERNST SIMONSON, C. A. MCKINLAY, AND AUSTIN HENSCHEL.

From the Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, Minn.

In a previous communication,[1] we reported, for 12 normal men, the effect of meals on electrocardiograms recorded 30 and 60 minutes later. Most of the electrocardiographic functions, especially the T-wave, showed sta-

* This work was supported by the Sugar Research Foundation, Inc., N.Y., and the National Dairy Council.

1 Simonson, E., Alexander, H., Henschel, A., and Keys, A., *Am. Heart J.*, 1946, **32**, 202.

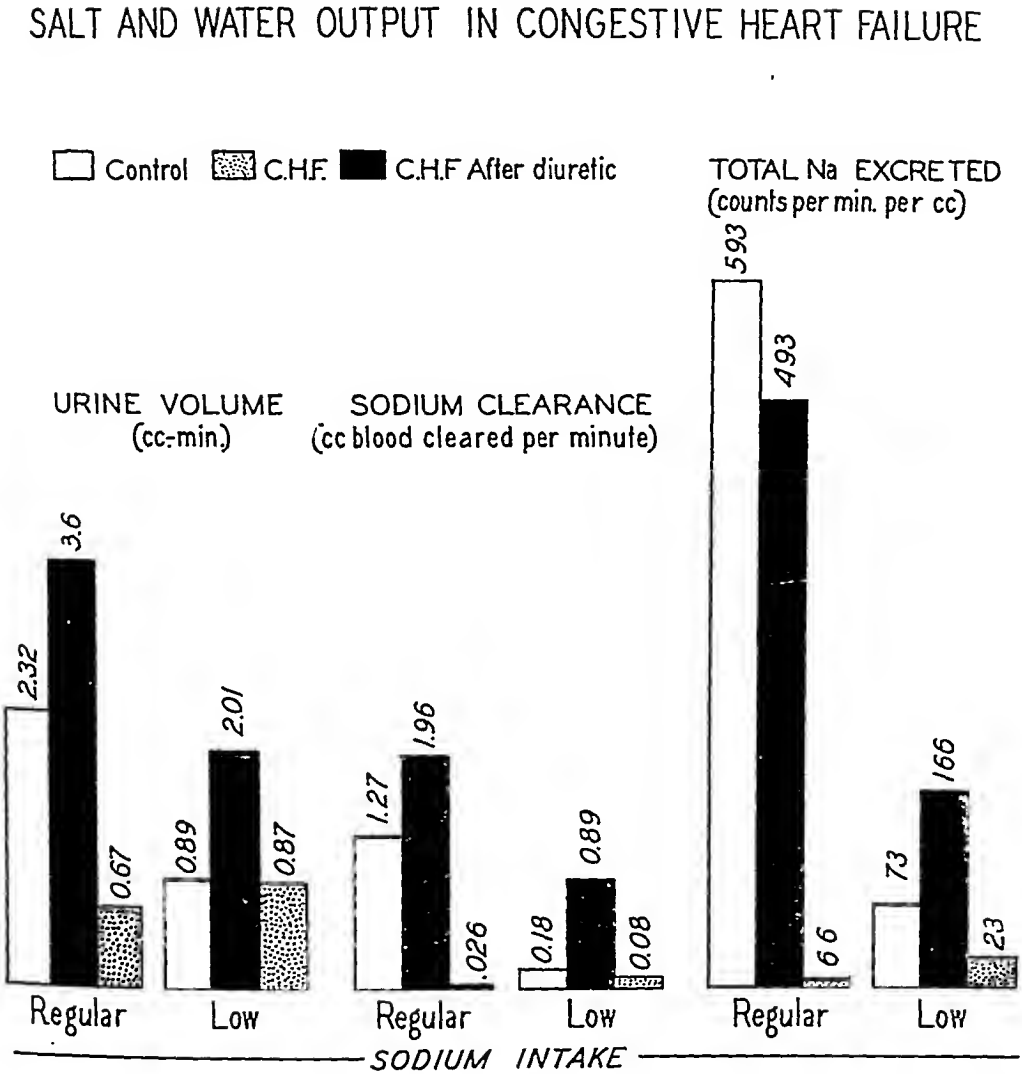


FIG. 1.

A comparison of the urine output, sodium clearance, and total sodium excretion on regular and low sodium diets in a congestive heart failure patient with and without a diuretic and a control subject. The heart failure patient was on a regular diet for 6 days and on a salt-free diet for 9 days, without a diuretic being administered, and 20 days during which 10 injections of a diuretic were given. The control subject was on a salt-free diet for 9 days (with only the last four used in the calculations) and on a regular diet 10 days.

identical conditions of salt and water intake. With mercurial diuresis the congestive heart failure patients' excretion of sodium was practically identical with the control and the excretion of water was greater (Fig. 1). In addition to the above data it was noted that: (a) The feces and vomitus counts were low and close to background. (b) The ascitic fluid and edema fluid were not appreciably different from the blood serum levels. (The removal of 1500 cc of ascitic fluid yielded as much sodium as would be excreted during 20 days without a diuretic). (c) In the control subject the blood serum Na^{22} concentration was found to decrease proportionately as the Na^{22} was excreted in the urine. In

study of the mechanism of edema this report is presented.

Two patients were followed simultaneously for 10 weeks. All voided and catheterized urine specimens and daily blood samples were collected during the first 3 weeks for the control and in the first 4 weeks for the heart failure patient. Samples of feces, vomitus, ascitic fluid and edema fluid were obtained when possible. Over 1500 5-minute counts were made. All samples were done in triplicate.

The congestive heart failure patient (elderly colored female) had hypertensive cardiovascular disease with acute failure and anasarca. This patient showed little if any improvement during her illness and died of acute heart failure 11 weeks after the study was started. The control was a young, colored female with vague arthraligic complaints of unknown cause. Both patients received the Na^{22} (less than 0.1 millicurie) as 200 cc of a 1% aqueous sodium chloride solution.

These patients were allowed to reach equilibrium with respect to the introduced sodium (more than 12 hours) before the results presented below were obtained. The determinations were made with a TA-Bl and scale-of-64. Technical Associates Geiger-Muller Counter.

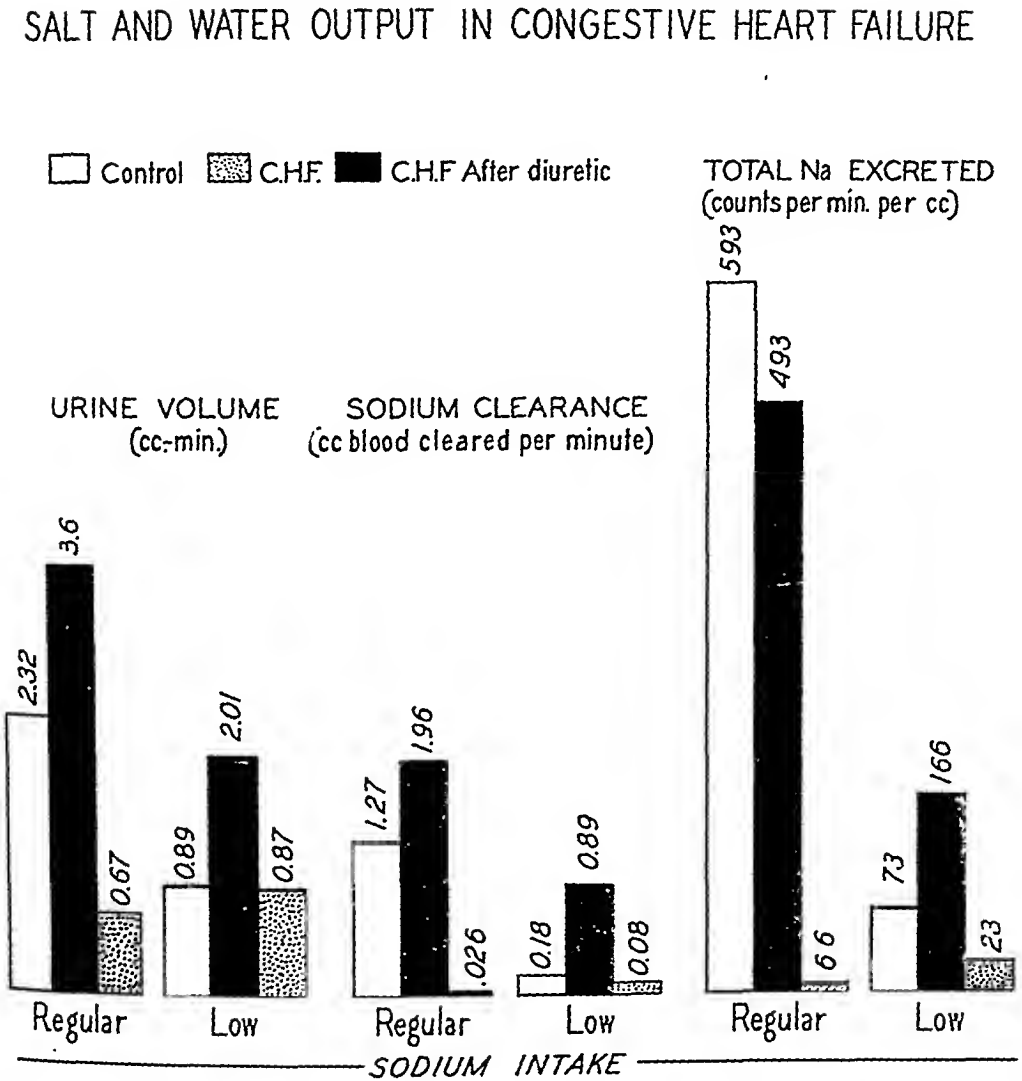
Results. The effect of sodium intake on the excretion of sodium and water in the normal, and in congestive heart failure with and without the use of a mercurial diuretic (sodium salt of methoxyoximercuripropylsuccinylurea with theophylline,—"Mercuhydrin," 1 cc I.M.)† was studied. It was found that the control required 5 days to reach a steady state of sodium excretion when placed on a low sodium diet. The results summarized in Fig. 1 are based on the succeeding 4 days. The congestive heart failure patient was on a low sodium diet (1.7 g NaCl or less, daily) without a diuretic for 9 days. During this time the mean sodium output of the control was 3.1 times that of the abnormal. This is to be expected because the available sodium was reduced in both patients. However, when it became readily available, such as when they were on a regular hospital diet (10 g or more NaCl) with salt *ad lib.*, the difference became quite marked. During 6 days (without a diuretic) for the abnormal and 10 days for the control the latter excreted 90 times as much sodium. In one 24-hour period this exceeded 200 times as much. That this was not due to the difference in urine volume is indicated by the fact that the latter were essentially the same on a low sodium intake and only 3.5 times greater in the control on a regular intake.

The individual urine concentrations of Na^{22} for the control were quite variable and no regular relationship existed between urine volume and Na^{22} concentration. A 1 cc dose of the diuretic produced no appreciable effect in this patient. The congestive heart failure patient's urine concentrations of Na^{22} were fixed at a very low level and in many instances the counts were at background. There was little relationship to volume, *e.g.*, a 10-fold change in volume was accompanied by no concentration changes. It might be added that the 6-day normal salt intake period for the congestive heart failure patient was initiated by a slow infusion of 1000 cc of physiological sodium chloride solution.

The question of selective sodium excretion after a mercurial diuretic is conclusively answered for this patient. During the salt-free period (20 days) 10 1-cc injections of the diuretic were given intramuscularly. The average response was a 7-fold increase in salt output (Fig. 1). When salt was made available for 6 days this response consisted of an average increase of 75 times. In one 24-hour period it reached a 160-fold increase. Following the diuretic, the water output increased only 2.3 times with salt restriction and 5.3 times with salt given *ad lib.* The sodium diuresis preceded the water diuresis by 2-4 hours. The diuretic became effective in less than 2 hours, reached a maximum in 4-6 hours, and lasted rather uniformly for almost 24 hours when given intramuscularly.

The question next arose as to the degree of normalcy approached by the treated congestive heart failure patient as determined by a comparison with the control under

† Courtesy Lakeside Laboratories, Milwaukee, Wis.



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The question next arose as to the degree of normalcy approached by the treated congestive heart failure patient as determined by a comparison with the control under

† Courtesy Lakeside Laboratories, Milwaukee, Wis.

tains glycine.

Methods. Seventeen male, adult albino rats, approximately 6 months of age and previously subsisting on Purina Dog Chow were used for this study. Creatinine and hippurate clearances were done to ascertain the control rates of glomerular filtration and renal plasma flow respectively. Approximately one week later, each rat received 50 mg of glycine per 100 g of body weight by stomach tube and the clearance studies were repeated immediately afterwards. The methods and calculations used in determining the clearances have been reported in a previous study.[7] All clearances have been expressed in cc per hour per 100 g of body weight.

TABLE I.
The Effect of Glycine on Creatinine and Hippurate Clearance of the Rat.

	Before Glycine			After Glycine		
Rat	U.V.*	C.C.†	H.C.‡	U.V.	C.C.	H.C.
27	1.75	30.4	180	2.9	41.5	101
25	3.60	40.8	212	4.4	36.2	149
93	4.95	39.0	90	5.2	41.5	144
72	3.70	39.4	139	2.0	28.0	182
92	5.0	48.8	175	3.5	39.2	157
57	4.0	39.5	202	4.7	40.0	146
10	0.60	18.6	91	4.75	44.0	185
12	2.4	34.8	146	3.55	38.6	89
98	2.3	34.6	108	2.80	28.6	119
35	3.6	34.4	158	2.40	40.8	142
29	4.9	28.5	115	0.90	20.8	64
88	3.9	43.7	160	2.10	24.7	108
21	3.6	38.7	134	3.30	19.8	122
09	3.5	38.8	128	2.75	39.7	128
20	4.6	32.5	73	2.25	45.7	81
77	4.6	37.0	138	2.85	36.8	191
93	4.55	50.5	152	3.05	42.8	138
Av.	3.62	37.1	141	3.14	35.8	132

* U.V. equals urine volume in cc per hour.
† C.C. equals creatinine clearance in cc per 100 g per hr.
‡ H.C. equals hippurate clearance in cc per 100 g per hour.

Results. Inspection of Table I indicates that no significant change occurred in either the creatinine or hippurate clearance of the rat following the ingestion of glycine. Thus the average creatinine clearance was 37.1 cc per hour before and 35.8 cc per hour after the ingestion of glycine. Likewise, the average hippurate clearance was 141 cc per hour before and 132 cc per hour after glycine had been given. The average urine volume per hour was 3.62 cc before and 3.14 cc after ingestion of glycine. This average decrease of 13% is perhaps of questionable significance.

It should be noted that Pitts[5] also found no increase in the creatinine clearance of dogs after heavy glycine feeding, nor could Beyer *et al.*[6] observe an increase in the dog's creatinine clearance after feeding of such essential amino acids as tryptophane, leucine, isoleucine or valine. Finally it should be emphasized that the results of this present study only indicate that *excess* feeding of glycine appears to have no effect on renal hemodynamics. It is conceivable that were glycine to be reduced abnormally in the blood, changes in renal hemodynamics might occur.

Conclusion. The administration of glycine to the rat had no demonstrable effect on either its rate of glomerular filtration or its renal blood flow.

The author wishes to express his thanks to Barbara Trousdale and Frances Greenberg for their technical assistance.

[7] Friedman, M., *Am. J. Physiol.*, 1947, to be published.

15668

Differentiation of Soy Bean Antitryptic Factors.

DONALD E. BOWMAN.

From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis.

In a preliminary report, the author called attention to a water-soluble antitryptic fraction of navy beans which can be readily precipitated with alcohol. Although a large

the congestive heart failure patient the presence of edema when the sodium was injected made similar calculations impossible. (d) The Na^{22} was 50% excreted by the control subject in 12 days and had disappeared in 10 weeks. In the congestive heart failure only 45% had been excreted in 10 weeks.

Discussion. The urine concentration of sodium is somewhat dependent upon the blood concentration at the time the urine is being formed. The exact relationship is unknown. Sodium clearances were employed to express this relationship. It must be remembered, however, that the term "clearance" cannot be used in the strict sense for sodium since the blood is less than 1% cleared of sodium during a single passage through the kidney. These patients formed urine of similar maximal concentration throughout the experiment. In the control this was equal to the blood concentration. In the congestive heart failure case it was lower but approached the blood level as the latter declined. It appears that the sodium concentrating ability in the congestive heart failure patient is fixed and not related to the blood level, whereas marked variations were possible in the normal.

Summary. The use of a radioactive tracer, Na^{22}, on an apparently normal control and a congestive heart failure patient showed a marked difference in respect to the behavior of this substance. (1) The control patient excreted an average of 90 times as much sodium as the congestive heart failure patient when both were given salt *ad lib.* (2) A mercurial diuretic resulted in an increase in sodium excretion of 75-fold when the congestive heart failure patient was receiving salt *ad lib.* (3) A reduction of salt excretion occurred in both when salt intake was restricted. However, the use of the diuretic still resulted in a 7-fold increase in the output of sodium in the congestive heart failure patient. (4) Other significant findings are presented.

The valuable technical assistance of Miss Shirley Wiedereeht and Messrs. G. Morgavi, F. M. Parks, J. Fruthaler, and R. Schramel is gratefully acknowledged.

15667

Effect of Glycine Feeding on Renal Hemodynamics of the Rat.*

MEYER FRIEDMAN.

From the Harold Brunn Institute for Cardiovascular Research, Mt. Zion Hospital, San Francisco.

The feeding of excess protein or its constituent amino acids has been found generally to increase the urea clearance in man[1,2] and in dog.[3,4] However, other than the studies of Van Slyke *et al.*,[3] little is known about the effect of these substances on the renal blood flow. Moreover, some investigators[5,6] have observed that the creatinine clearance did not rise after the ingestion of excess amino acids. It seemed important then, to study the effect of the common amino acid, glycine on the renal hemodynamics of the rat. This amino acid was employed because most investigators[2,4] are agreed that whatever effect protein does have on renal function, resides in its amino acid content which in turn almost invariably con-

* Aided by a grant from the Dazian Foundation for Medical Research.

1 Addis, T., and Drury, D. R., *J. Biol. Chem.*, 1923, **55**, 629.

2 Longley, L. P., and Miller, M., *Am. J. Med. Sci.*, 1942, **203**, 253.

3 Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A., *Am. J. Physiol.*, 1934, **110**, 387.

4 Pitts, R. F., *J. Nutrition*, 1935, **9**, 657.

5 Pitts, R. F., *Am. J. Physiol.*, 1944, **140**, 535.

6 Beyer, K. H., Wright, L. D., Russo, H. F., Skeggs, H. R., and Patch, E. A., *Am. J. Physiol.*, 1946, **146**, 330.

tains glycine.

Methods. Seventeen male, adult albino rats, approximately 6 months of age and previously subsisting on Purina Dog Chow were used for this study. Creatinine and hippurate clearances were done to ascertain the control rates of glomerular filtration and renal plasma flow respectively. Approximately one week later, each rat received 50 mg of glycine per 100 g of body weight by stomach tube and the clearance studies were repeated immediately afterwards. The methods and calculations used in determining the clearances have been reported in a previous study.[7] All clearances have been expressed in cc per hour per 100 g of body weight.

TABLE I.
The Effect of Glycine on Creatinine and Hippurate Clearance of the Rat.

	Before Glycine			After Glycine		
Rat	U.V.*	C.C.†	H.C.‡	U.V.	C.C.	H.C.
27	1.75	30.4	180	2.9	41.5	101
25	3.60	40.8	212	4.4	36.2	149
93	4.95	39.0	90	5.2	41.5	144
72	3.70	39.4	139	2.0	28.0	182
92	5.0	48.8	175	3.5	39.2	157
57	4.0	39.5	202	4.7	40.0	146
10	0.60	18.6	91	4.75	44.0	185
12	2.4	34.8	146	3.55	38.6	89
98	2.3	34.6	108	2.80	28.6	119
35	3.6	34.4	158	2.40	40.8	142
29	4.9	28.5	115	0.90	20.8	64
88	3.9	43.7	160	2.10	24.7	108
21	3.6	38.7	134	3.30	19.8	122
09	3.5	38.8	128	2.75	39.7	128
20	4.6	32.5	73	2.25	45.7	81
77	4.6	37.0	138	2.85	36.8	191
93	4.55	50.5	152	3.05	42.8	138
Av.	3.62	37.1	141	3.14	35.8	132

* U.V. equals urine volume in cc per hour.
† C.C. equals creatinine clearance in cc per 100 g per hr.
‡ H.C. equals hippurate clearance in cc per 100 g per hour.

Results. Inspection of Table I indicates that no significant change occurred in either the creatinine or hippurate clearance of the rat following the ingestion of glycine. Thus the average creatinine clearance was 37.1 cc per hour before and 35.8 cc per hour after the ingestion of glycine. Likewise, the average hippurate clearance was 141 cc per hour before and 132 cc per hour after glycine had been given. The average urine volume per hour was 3.62 cc before and 3.14 cc after ingestion of glycine. This average decrease of 13% is perhaps of questionable significance.

It should be noted that Pitts[5] also found no increase in the creatinine clearance of dogs after heavy glycine feeding, nor could Beyer *et al.*[6] observe an increase in the dog's creatinine clearance after feeding of such essential amino acids as tryptophane, leucine, isoleucine or valine. Finally it should be emphasized that the results of this present study only indicate that *excess* feeding of glycine appears to have no effect on renal hemodynamics. It is conceivable that were glycine to be reduced abnormally in the blood, changes in renal hemodynamics might occur.

Conclusion. The administration of glycine to the rat had no demonstrable effect on either its rate of glomerular filtration or its renal blood flow.

The author wishes to express his thanks to Barbara Trousdale and Frances Greenberg for their technical assistance.

[7] Friedman, M., *Am. J. Physiol.*, 1947, to be published.

15668

Differentiation of Soy Bean Antitryptic Factors.

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In a preliminary report, the author called attention to a water-soluble antitryptic fraction of navy beans which can be readily precipitated with alcohol. Although a large

part of the trypsin-inhibiting material of soy beans is not precipitated with alcohol, it is precipitated with acetone.[1] It was further pointed out that soy and other beans as well as navy beans do, however, contain a trypsin-inhibiting fraction which can be repeatedly reprecipitated with alcohol.[2]

Kunitz soon crystallized this latter factor from soy beans and described some of its properties.[3,4] He found it to be a crystalline globulin which can be further recrystallized from 20% alcohol. Further comment regarding the occurrence of this factor is hardly necessary; however, the trypsin-retarding fraction of soy beans, which is quite soluble in 60% alcohol but insoluble in acetone, would appear to deserve further attention. Following the publication of our description of this fraction,[1] it was found that a note by Ham and Sandstedt[5] had appeared shortly before referring to what appears to be this material, and we wish to acknowledge their paper. They indicated that this antitryptic substance is destroyed upon treating an aqueous extract of the beans with 60% alcohol, and they found no inhibiting action in aqueous extracts of soy beans previously treated with 45% alcohol. Other properties given also differentiate it from the antitryptic substance which is found in alcohol precipitates of aqueous extracts of soy beans. These investigators may have discarded the latter substance with the kaolin used to remove proteins since it will adsorb on kaolin.

A number of soy bean preparations having antitryptic properties have been used in various investigations. Employing an acetone precipitate of an aqueous extract, Ham, Sandstedt, and Mussehl pointed out that the proteolytic-inhibiting material has a retarding effect on the growth of chicks.[6] Klose, Hill and Fevold observed similar growth effects and indicated that the substance which they employed is nondialyzable, can be precipitated by salts, is inactivated by heat, and appears to be a protein which is concentrated in the acid-soluble (pH 4.2) fraction of soy bean protein.[7] Tagnon and Soulier, employing the inhibitor obtained by acetone precipitation of aqueous extracts as well as the crystalline material of Kunitz, observed marked anticoagulant activity on human whole blood and plasma.[8] Employing the acetone-insoluble and alcohol-insoluble fractions, the author has been unable to show a consistent influence on trypsin shock.[2] Thompson has indicated that the alcohol-insoluble factor of navy beans does not modify anaphylactic shock produced by egg albumin.[9]

Unless definite precautions are taken, crude soy bean antitryptic preparations may consist of varying proportions of at least 2 factors. This occurs especially when acetone is used to precipitate the active material from an aqueous extract. The object of the present report is to call attention to the difference between the soy bean antitryptic substance which can be extracted with water and precipitated with alcohol, called the alcohol-insoluble factor, and the active material which can be extracted with 60% alcohol and precipitated with acetone, called the acetone-insoluble factor. Both are soluble in water.

Methods. Relative inhibition of tryptic activity was estimated by adding the experimental material to triplicate casein digestion preparations, and the degree of digestion was compared with that of the same number of control uninhibited preparations of equal volume, run simultaneously. With final concentrations of 0.01% of crude trypsin and 3.1% casein, approximately half of the casein was digested at 38°C in one hour in the absence of inhibiting agents. The actual degree of digestion was determined by the increase in

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2 Bowman, D. E., *Fed. Proc.*, 1945, **4**, 84.

3 Kunitz, M., *Science*, 1945, **101**, 668.

4 Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

5 Ham, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

6 Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

7 Klose, A. A., Hill, B., and Fevold, H. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 10.

8 Tagnon, H. J., and Soulier, J. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 440.

9 Thompson, R. L., personal communication.

TABLE I.
Comparison of Antitryptic Preparations.

Treatment and portion used	% of normal uninhibited digestion in the presence of inhibiting substances		
	Acetone-insoluble fraction, 0.25%*	Alcohol-insoluble fraction, 0.04%*	Crystalline material, 0.04%*
Untreated	49.9	52.5	46.9
Material insoluble in 60% alcohol	103.0	54.6	47.3
Untreated	45.5	54.2	46.8
Material insoluble in a solution 40% saturated with ammonium sulfate	102.0	56.6	49.5
Untreated	44.8	51.0	47.0
Material soluble in 2.5% trichloracetic acid	46.0	97.0	99.4

* Concentration of untreated trypsin inhibiting fraction in the casein digestion mixtures. The concentration of the treated material would have been the same except for the influence of the treatment.

the refractive index[10] of the filtrate following the isoelectric precipitation of undigested casein with a constant amount of acetic acid. In all cases triplicate unincubated negative controls were run in which the casein was precipitated before the trypsin was added and the filtrate was employed immediately. Triplicate values ordinarily agreed quite well throughout.

The material referred to as the acetone-insoluble factor was prepared by first extracting 100 g of finely ground untreated soy beans with one liter of 60% ethyl alcohol. The active material was then precipitated from the centrifuged and filtered alcohol extract by adding 2 volumes of acetone. The precipitate was centrifuged down and immediately dried in a vacuum desiccator. The majority of such preparations were further redissolved and heated in 2.5% trichloracetic acid at 98°C for 5 minutes. After centrifuging down and discarding the precipitate the active substance was reprecipitated from the neutralized solution with 90% acetone.

The alcohol-insoluble factor was obtained by extracting 100 g of finely ground untreated soy beans with one liter of water overnight, after adjusting to pH 4. After centrifuging, the supernatant aqueous extract was precipitated by adding alcohol to 60% concentration. The precipitate was centrifuged and dried in a vacuum desiccator.

Results. Numerous preparations of the 2 factors were employed and compared with a crystalline preparation provided by Dr. Kunitz. Typical results are given in Table I. As may be expected from the methods of preparation, the solubilities of the 2 factors in 60% alcohol are quite different. This can be shown more objectively by comparing the trypsin-inhibiting capacity of a suitable weighed amount of the untreated substance with the inhibiting capacity of an equal amount after extraction with 100 parts of 60% alcohol by weight. The acetone-insoluble factor appears to completely dissolve, and no activity is apparent after centrifuging and drying any possible insoluble material and adding it to casein digestion mixtures. Under the same conditions the alcohol-insoluble factor and the crystalline material appeared to be insoluble and, as judged by trypsin-inhibiting capacity, essentially entirely recovered in the undissolved material.

The acetone-insoluble factor does not precipitate when present in 1% concentration in a solution 40% saturated with ammonium sulfate. After centrifuging down any slight amount of insoluble material present, the latter was redissolved and the pH adjusted to that of the digestion mixture. No antitryptic activity was observed. In contrast, under similar conditions the alcohol-insolu-

[10] Robertson, T. B., *J. Biol. Chem.*, 1912, **12**, 23.

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protection against the intoxication ensuing once the bacteria have liberated their antigens into the bloodstream. Indeed the toxicity of *Shigella*[4] and *Salmonella*[5] vaccines employed for prophylactic immunization has been a complicating factor; and the excessive toxicity of *Shigella* vaccines has constituted a hazardous accompaniment to their successful use, even when neurotoxin-free vaccines are employed. It follows that the problem of therapeutics directed specifically at preformed endotoxin is of considerable clinical significance.

The only pharmacological (as distinguished from immunological) agents known to lessen experimentally the toxic effects of these antigens were sulfonamide drugs, whose effectiveness, however, in this respect was low, protecting mice against only a few lethal doses of the endotoxin.

Accordingly, over several years we have undertaken a search for pharmacological agents which might be more effective than sulfanilamide in counteracting the effects of the endotoxin. We have tested the compounds and combinations of compounds listed below* for protective effects against the endotoxin in mice. The rationale governing their selection for trial need not be given here. It may be noted, however, that nearly

[4] Goebel, W. F., Perlman, E., and Binkley, F., *Science*, 1944, **99**, 412.

[5] Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity*, Williams & Wilkins, Baltimore, 3rd Ed., 1946, p. 1553.

* (1) N¹-acetylsulfanilamide; (2) N⁴-acetylsulfanilamide; (3) *p*-aminoazobenzene; (4) o-aminobenzene sulfonic acid; (5) *p*-aminobenzoic acid; (6) anthranilic acid; (7) arginine; (8) arginine, cysteine, methionine; (9) ascorbic acid; (10) ascorbic acid, cortical adrenal extract; (11) ascorbic acid, guanine, thiamine, methionine; (12) ascorbic acid, cysteine, glycine, methionine, *p*-aminobenzoic acid, calcium gluconate, sodium acetate; (13) ascorbic acid, glutathione, (14) *p*-aminophenol; (15) atropine; (16) atropine, ergotoxin; (17) benzedrine; (18) benzenesulfonylamide; (19) bulbocapnine; (20) butanediol; (21) calcium gluconate; (22) carrot diet; (23) camphor; (24) catechol; (25) catechol (acetylated); (26) casein hydrolysate; (27) casein hydrolysate, ascorbic acid, adrenal cortical extract, sodium chloride; (28) choline; (29) coramine; (30) coramine, sulfanilamide, calcium gluconate; (31) coramine, atropine; (32) coramine, metrazol; (33) concanavalin; (34) cortical adrenal extract; (35) cortical adrenal extract, amino acid mixture; (36) cysteine; (37) cysteine, methionine; (38) cysteine, methionine, sodium acetate; (39) cysteine, glycine, yeast, nucleic acid, arginine, sodium acetate; (40) 4,4-diaminodiphenyl sulfone; (41) diaminophenol; (42) diacetyl; (43) diphenylsulfone; (44) ergotoxin; (45) glycolic acid; (46) glycine; (47) glutathione; (48) glutathione, monoethalolamine salt of ascorbic acid; (49) heparin; (50) hesperidine; (51) hesperidine, ascorbic acid; (52) histamine; (53) histamine, tryptophane, ascorbic acid, methionine; (54) histamine, tryptophane, methionine; (55) histidine; (56) hydroquinone; (57) *p*-hydroxybenzoic acid, hydroquinone triacetate, hydroquinone; (58) insulin; (59) insulin, dextrose; (60) liver (Difco); (61) liver (Lederle, 6E, 6C, 9C); (62) liver, sulfadiazine; (63) methionine; (64) metanilamide; (65) metrazol; (66) nicotinic acid; (67) o-nitrophenol; (68) *p*-nitrosophenol; (69) *p*-nitrobenzoic acid; (70) yeast nucleic acid; (71) nupercaine; (72) orcinol; (73) *o*-phenetidine; (74) *p*-phenetidine; (75) o-phenylenediamine; (76) picrotoxin; (77) quinone; (78) resorcinol; (79) sodium acetate; (80) sodium chloride; (81) sodium formaldehyde sulfoxylate; (82) sulfanilic acid; (83) thiamine; (84) thiouracil; (85) tryptophane; (86) tyrosine; (87) yeast, liver diet; (88) yeast extract (Difco).

The tests in most instances consisted of injecting groups of 6 mice each with intraperitoneal doses of 2-LD_{50} of the endotoxin. Previously to or simultaneously with the injection of the endotoxin, the mice received by repeated intraperitoneal injections or by stomach tube, the test chemicals in sub-toxic doses. Survival of the mice at the end of 24 hours after injection of the endotoxin served as the index of reduction in toxicity brought about by the test chemicals.

The mice used in these tests and in those experiments presented below were all young males of a Swiss line in the weight range of 18-21 g, obtained from the Rockland Farms. The animals were maintained during the experimental period, unless otherwise specified on a commercial mouse diet and tap water *ad libitum*.

The methods used in preparing the endotoxin material from *Salmonella typhimurium* employed in the above tests have been previously described.[6]

[6] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 187.

ble factor and the crystalline material give a definite precipitate and, in each case, the trypsin-inhibiting capacity of the precipitate indicates essentially complete recovery.

Although crystalline soy bean antitrypsin is precipitated when heated in 2.5% trichloracetic acid as pointed out by Kunitz,[3] the acetone-insoluble factor can be almost quantitatively recovered from the filtrate of such a solution. This is indicated by the final antitryptic capacity retained after so treating a weighed amount of the material. After heating this inhibitor, in 2% concentration, in 2.5% trichloracetic acid at 98°C for 5 minutes only a very slight precipitate forms. After centrifuging and discarding this precipitate and neutralizing the supernatant solution, the active material can be almost completely recovered by precipitating with 90% acetone.

Upon treating the alcohol-insoluble factor or the crystalline material in a similar manner, an appreciable precipitate is seen in the trichloracetic acid solution and the amount of antitryptic substance obtained from the neutralized filtrate by precipitating with 90% alcohol is insignificant.

A typical preparation of the acetone-insoluble fraction was found to contain approximately 1% nitrogen and gave only faintly positive biuret and Millon tests. Further observations dealing with the nature of the active material are in progress. Its solubility characteristics and a phosphorus content of about 5% in the present preparations suggests that some attention be given to phosphorus-containing substances.

Summary. In a number of respects, the trypsin-inhibiting factor of soy beans which may be extracted with 60% alcohol and precipitated with acetone differs from the antitrypsin which can be extracted with water and precipitated with 60% alcohol. These substances may be differentiated on the basis of their solubilities in a solution 40% saturated with ammonium sulfate, in 2.5% trichloracetic acid as well as in alcohol.

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Pharmacological Protection Against *Salmonella* Endotoxin and Certain Other Poisons.

PAUL A. ZAHL, M. L. DRASHER, AND S. H. HUTNER.
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In previous work sulfonamides were found effective to a limited degree in counteracting the systemic toxicity of certain typical endotoxins of Gram-negative bacteria.[1] Analysis of this action of sulfonamides has led to the conclusion, reported here, that the efficacy of sulfonamides in this respect depends on their goitrogenic activity, and posed again the problem of devising an efficient therapy directed against this class of poisons.

The endotoxin-containing somatic (O) antigens of Gram-negative bacteria are frequently the principal source of damage in infections by these organisms.[2] In contrast to the classical bacterial exotoxins, antibodies for these somatic antigens have little antitoxic potency.[3] The action of these endotoxins is usually aborted by checking the multiplication of the bacteria either by means of previous immunization with appropriate vaccines or, when the infection has gained a foothold, by administration of antibiotics such as streptomycin. Although these measures are often dramatically effective when directed against the primary infection, they afford little or no

1 Hutner, S. H., and Zahl, Paul A., *Science*, 1942, **96**, 563; Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 285.

2 Zahl, Paul A., Starr, M. P., and Hutner, S. H., *Am. J. Hyg.*, 1945, **41**, 41.

3 Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, 1944, **39**, 189.

protection against the intoxication ensuing once the bacteria have liberated their antigens into the bloodstream. Indeed the toxicity of *Shigella*[4] and *Salmonella*[5] vaccines employed for prophylactic immunization has been a complicating factor; and the excessive toxicity of *Shigella* vaccines has constituted a hazardous accompaniment to their successful use, even when neurotoxin-free vaccines are employed. It follows that the problem of therapeutics directed specifically at preformed endotoxin is of considerable clinical significance.

The only pharmacological (as distinguished from immunological) agents known to lessen experimentally the toxic effects of these antigens were sulfonamide drugs, whose effectiveness, however, in this respect was low, protecting mice against only a few lethal doses of the endotoxin.

Accordingly, over several years we have undertaken a search for pharmacological agents which might be more effective than sulfanilamide in counteracting the effects of the endotoxin. We have tested the compounds and combinations of compounds listed below* for protective effects against the endotoxin in mice. The rationale governing their selection for trial need not be given here. It may be noted, however, that nearly

[4] Goebel, W. F., Perlman, E., and Binkley, F., *Science*, 1944, **99**, 412.

[5] Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity*, Williams & Wilkins, Baltimore, 3rd Ed., 1946, p. 1553.

* (1) N1-acetylsulfanilamide; (2) N4-acetylsulfanilamide; (3) *p*-aminoazobenzene; (4) *o*-aminobenzene sulfonic acid; (5) *p*-aminobenzoic acid; (6) anthranilic acid; (7) arginine; (8) arginine, cysteine, methionine; (9) ascorbic acid; (10) ascorbic acid, cortical adrenal extract; (11) ascorbic acid, guanine, thiamine, methionine; (12) ascorbic acid, cysteine, glycine, methionine, *p*-aminobenzoic acid, calcium gluconate, sodium acetate; (13) ascorbic acid, glutathione, (14) *p*-aminophenol; (15) atropine; (16) atropine, ergotoxin; (17) benzedrine; (18) benzenesulfonylamide; (19) bulbocapnine; (20) butanediol; (21) calcium gluconate; (22) carrot diet; (23) camphor; (24) catechol; (25) catechol (acetylated); (26) casein hydrolysate; (27) casein hydrolysate, ascorbic acid, adrenal cortical extract, sodium chloride; (28) choline; (29) coramine; (30) coramine, sulfanilamide, calcium gluconate; (31) coramine, atropine; (32) coramine, metrazol; (33) concanavalin; (34) cortical adrenal extract; (35) cortical adrenal extract, amino acid mixture; (36) cysteine; (37) cysteine, methionine; (38) cysteine, methionine, sodium acetate; (39) cysteine, glycine, yeast, nucleic acid, arginine, sodium acetate; (40) 4,4-diaminodiphenyl sulfone; (41) diaminophenol; (42) diacetyl; (43) diphenylsulfone; (44) ergotoxin; (45) glycolic acid; (46) glycine; (47) glutathione; (48) glutathione, monoethalolamine salt of ascorbic acid; (49) heparin; (50) hesperidine; (51) hesperidine, ascorbic acid; (52) histamine; (53) histamine, tryptophane, ascorbic acid, methionine; (54) histamine, tryptophane, methionine; (55) histidine; (56) hydroquinone; (57) *p*-hydroxybenzoic acid, hydroquinone triacetate, hydroquinone; (58) insulin; (59) insulin, dextrose; (60) liver (Difco); (61) liver (Lederle, 6E, 6C, 9C); (62) liver, sulfadiazine; (63) methionine; (64) metanilamide; (65) metrazol; (66) nicotinic acid; (67) *o*-nitrophenol; (68) *p*-nitrosophenol; (69) *p*-nitrobenzoic acid; (70) yeast nucleic acid; (71) nupercaine; (72) orcinol; (73) o-phenetidine; (74) *p*-phenetidine; (75) *o*-phenylenediamine; (76) picrotoxin; (77) quinone; (78) resorcinol; (79) sodium acetate; (80) sodium chloride; (81) sodium formaldehyde sulfoxylate; (82) sulfanilic acid; (83) thiamine; (84) thiouracil; (85) tryptophane; (86) tyrosine; (87) yeast, liver diet; (88) yeast extract (Difco).

The tests in most instances consisted of injecting groups of 6 mice each with intraperitoneal doses of 2-LD_{50} of the endotoxin. Previously to or simultaneously with the injection of the endotoxin, the mice received by repeated intraperitoneal injections or by stomach tube, the test chemicals in sub-toxic doses. Survival of the mice at the end of 24 hours after injection of the endotoxin served as the index of reduction in toxicity brought about by the test chemicals.

The mice used in these tests and in those experiments presented below were all young males of a Swiss line in the weight range of 18-21 g, obtained from the Rockland Farms. The animals were maintained during the experimental period, unless otherwise specified on a commercial mouse diet and tap water *ad libitum*.

The methods used in preparing the endotoxin material from *Salmonella typhimurium* employed in the above tests have been previously described.[6]

[6] Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 187.

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0.75	3.0 "	20	95
0.75	C	20	35
2.00	3.0 "	24	60
2.00	C	25	12

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It is evident from the data listed in Table I that thiouracil affords a degree of protection against the toxic effects of the endotoxin. In other exploratory experiments in which only a few hours elapsed between the time of the first dose of thiouracil and the injection of the endotoxin, very little protective effect could be observed, due presumably to the slowness of interference with thyroid function by thiouracil. Also, when the pretreatment with thiouracil was extended to 7 days before the injection of the endotoxin, little if any protective effect was observed, due possibly to the general debilitating effects of sustained thiouracil treatment, as shown by the fact that mice so treated and with such relatively high dosages failed to gain weight at the normal rate.

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A similar type of experiment was performed with colchicine. This poison is presumed to

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TABLE II.

Dose of endotoxin (intraper.) mg	Dose of thiouracil (intraper.) mg	No. of animals	Degree of tumor hemorrhage*
0.10	3.0 (×6)	18	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 ++
0.10	(control)	14	+++ +++ +++ +++ ++++ ++++ ++++ ++++ ++++ ++++ 0 0

* Each designation below indicates the degree of tumor hemorrhage induced in each mouse. 0 = no hem.; + = slight hem.; ++ = considerable hem.; +++ = marked hem.; ++++ = maximal hem.

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16	—	3.0 mg (×6)	81	53
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Effect of Temperature on the Toxicity of the Endotoxin. From other studies it has been concluded that the effects of thiouracil (on anoxia, etc.) are attributable to a general lowering of metabolic rate via interference with thyroid gland activity.[8] If such an action accounts for the thiouracil effect on the toxicity of the endotoxin and dichloroethane, then a similar effect on these poisons should be observed by altering general body metabolism in other ways. Accordingly, an experiment was set up in which groups of mice were kept at various temperatures for the 24-hour period following injection of the endotoxin. The results of this experiment are summarized in Table IV. Clearly, mice maintained at high environmental temperatures are more susceptible to the toxicity of the endotoxin than those kept at lower temperatures.

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Air temperature of incubator, °C	Dose of endotoxin (intraper.)	No. of mice	% survival
14	0.75 mg	19	74
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[7] Ludford, R. J., *J. Nat. Cancer Inst.*, 1945, **6**, 89.

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[13] Miller, C. P., and Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 18.

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15670 P

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The fluid inside the capsule (±5 cc) was inoculated with the fungus under investigation, while the outside fluid was inoculated with a recently isolated strain of virulent tubercle bacilli. While a rapid and easy exchange of metabolic product occurred through the alundun wall, the coarse fungus filaments did not as a rule penetrate it and invade the outer fluid. In the few cases in which this happened, the filtrate from the fungus growth was tested for the presence of growth inhibiting substances.

Of a large number of fungi, recently isolated from a variety of sources, only one, a Fusarium, sp., inhibited growth of the tubercle bacillus.

The inhibiting substance passes through fritted glass filters and Mandler filters but is partially inactivated by passage through Seitz filters. The crude filtrate is active up to a dilution of 1/20 and withstands heating to 100° for 15 minutes without loss of activity.

Experiments are now in progress to isolate the active principle and determine its activity *in vivo*.

C. D. Sherbakoff, Head Dept. of Plant Pathology, The University of Tennessee, Agricultural Experiment Station, has kindly identified the Fusarium as *Fusarium scirpi* Lamb. et Fautr. v. accuminatum (Ell. et Ev.) Wr. Wollenweber in his monograph on Die Fusarien, Z. f. Parasitenkunde, Berlin, 1931, places this Fusarium as No. 930 in a list of 1100.

15671 P

Urinary Excretion of Orally Administered Pteroylglutamic Acid.*

RUTH STEINKAMP, CARROLL F. SHUKERS, JOHN R. TOTTER, AND PAUL L. DAY.
From the Department of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock.

Introduction. Few studies on the urinary excretion of pteroylglutamic acid have been reported.[1-4] No published information concerning the excretion of this vitamin after single large doses is available. Consequently, we are reporting results of such studies on 9 normal subjects and 9 hospitalized patients representing assays on 54 24-hour urine samples.

Methods. Since the primary purpose of the study was to determine the recovery of the free pteroylglutamic acid, no resort was made to the use of enzymes to liberate any conjugated vitamin.

One or more preliminary 24-hour urine samples were obtained in all but 2 instances. The test dose of synthetic pteroylglutamic acid was given orally (5.0, 5.1, 10 or 16 mg) and samples of urine were collected at varying intervals for the 24-hour period immediately following. The samples were preserved under benzene and aliquots were suitably diluted for determining the PGA microbiologically[5] with *Streptococcus faecalis*

* Research paper No. 830, Journal Series, University of Arkansas. This work was supported in part by a grant-in-aid from the Nutrition Foundation, Inc., New York. We are indebted to the Lederle Laboratories, Inc., for supplies of synthetic pteroylglutamic acid ("Folvite").

1 Denko, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Friedman, T. E., and Youmans, J. B., *Arch. Biochem.*, 1946, **10**, 33.

2 Denko, C. W., Grundy, W. E., Wheeler, N. C., Henderson, C. R., Berryman, G. H., Friedman, T. E., and Youmans, J. B., *Arch. Biochem.*, 1946, **11**, 109.

3 Wright, L. D., and Welch, A. D., *Science*, 1943, **98**, 179.

4 Johnson, B. C., Hamilton, T. S., and Mitchell, H. H., *J. Biol. Chem.*, 1945, **159**, 425.

5 Mitchell, H. K., and Snell, E. E., *The Univ. of Texas Publication*, 1941, No. 4137, 36.

TABLE I.
Preliminary Twenty-four-hour Urinary Excretion of Pteroylglutamic Acid.

Normal Subjects				Hospital Patients					
Subject No.	Sex	24-hour excretion level pteroylglutamic acid, μg	Avg, μg	Subject No.	Sex	Diagnosis	24-hour excretion level pteroylglutamic acid, μg	Avg, μg
1	M	2.4; 2.0	2.65	10	M	Pituitary deficiency	2.3; 5.5	3.9
2	M	2.4; 3.4	2.9	11	M	Lymphatic leukemia	0.9; 1.3; 1.9; 1.4; 1.0; 2.3; 0.8	1.3
3	F	6.0; 2.8; 3.4	4.07	12	F	Hemorrhagic anemia	1.7	1.7
4	F	3.0; 1.5	2.25	13	M	Myelogenous leukemia	1.0; 2.3; 1.8	1.7
5	M	2.0	2.0	14	M	Anemia	1.8	1.8
6	F	2.7; 1.5	2.1	15	F	Lymphosarcoma	8.5; 4.7; 2.3; 2.6; 1.0; 1.3; 6.9; 11.9	4.9
7	F	2.9	2.9	16	M	Anemia	0.9; 0.7	0.8
8	M	0.07	0.07	17	M	"		
9	F	2.0	2.0	18	M	Cardiac insufficiency		
		Mean	2.34 μg			Mean		2.3 μg
		Standard deviation	±1.08 μg			Standard deviation		±1.5 μg

(American Type Culture Collection No. 8043). Synthetic pteroylglutamic acid was used as the standard. Turbidimetric readings were made with a Coleman spectrophotometer.

Results. The assay values for the preliminary 24-hour periods are given in Table I. The mean value for 15 determinations on normal subjects was 2.34 $\gamma \pm 1.08\ \gamma$: that for the 24 studies on the hospitalized patients was 2.3 $\gamma \pm 1.5\ \gamma$. These values are in satisfactory agreement with results obtained by Denko *et al.*[1] and by Wright and Welch[2] on normal individuals.

Data on the rate of excretion of the test doses are given in Fig. 1. The data are presented in terms of the percentage of test dose excreted, since this appears to be independent of the size of the test dose used.

The curve is drawn through the cumulative average recoveries from the normal individuals for 4, 8 and 24 hours. The greater part of the excretion of the vitamin took place between the second and eighth hours following oral administration. The total 24-hour percentage recovery for the normal subjects averaged 28.5 (range 24.5-37.5%). With the exception of one patient who had received 5 mg of "Folvite"* 12 days previously, the total 24-hour recoveries for the 8 samples from hospital patients fell below 11% (range for all patients 1.75-26.9%). The diagnoses and the preliminary urinary pteroylglutamic acid excretion levels are presented in Table I. The surprisingly small percentage recoveries of the vitamin in these patients may indicate either a low degree of saturation, increased destruction, increased requirement, or the conjugation of pteroylglutamic acid before excretion. The data at hand do not permit a choice among these possibilities.

Summary. Fifty-four urine samples for 24-hour periods have been assayed microbiologically for free pteroylglutamic acid. For normal subjects the average 24-hour excretion was 2.34 γ. Following an oral dose of pteroylglutamic acid the mean percentage recovery was 28.5%. Although the hospitalized patients studied had a similar excretion level (average 2.3 γ for 24 hours) prior to dosage with pteroylglutamic acid, the per-

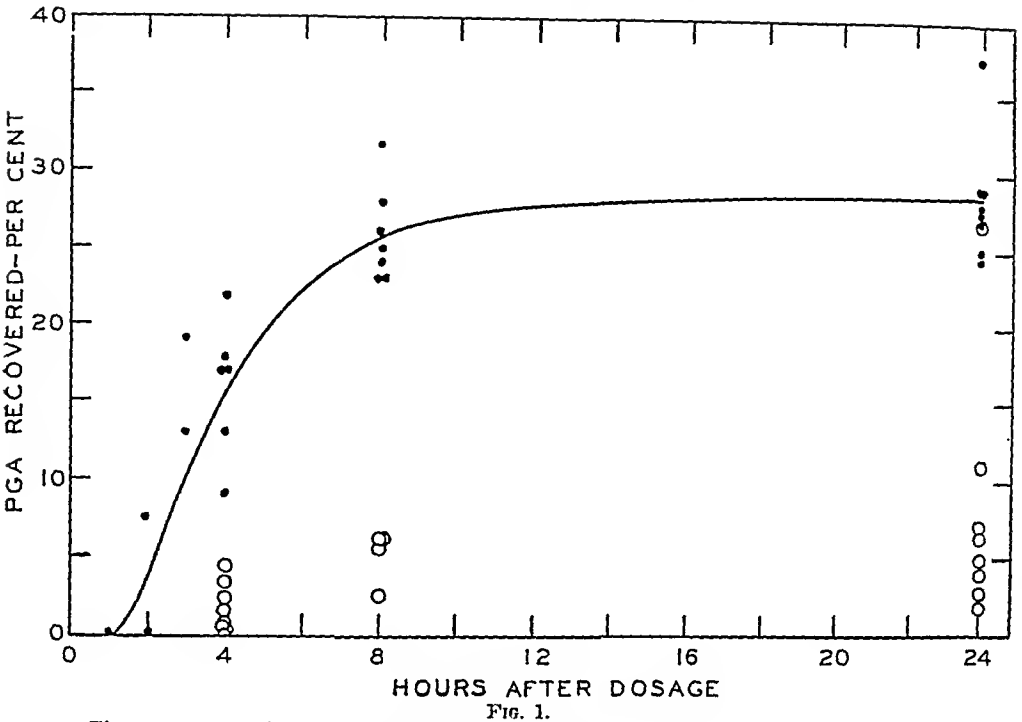

FIG. 1.

The percentage urinary recovery of pteroylglutamic acid after single oral doses of 5.0, 5.1, 10 or 10 mg. ●—Normal subjects; ○—hospital patients.

centage returns from single oral doses were much lower than in the normal subjects, a finding which is possibly indicative of a low degree of saturation.

15672 P

Effect of Folic Acid upon Primitive Erythrocytes *In vitro*.*

EDWIN E. HAYS.†

From the Department of Biochemistry, University of Vermont, College of Medicine, Burlington, Vermont.

Since the antianemic properties of pteroylglutamic acid (synthetic folic acid) were reported[1] there have been many studies to determine how this substance acts upon the bone marrow to stimulate the production of cells and to ascertain the relationship of pteroylglutamic acid to the antipernicious anemia principle. Efforts to demonstrate significant amounts of pteroylglutamic acid in highly potent antipernicious anemia liver fractions have failed.[2,3] Attempts made to liberate free pteroylglutamic acid from ground muscle treated with normal gastric juice according to the technic of Castle and Town-

* Aided by a grant from The Armour Laboratories, Armour and Company, Chicago, Ill.

† With the technical assistance of Miss Betty Paulsen, Mrs. Francena Galbraith, and Miss Constance Brownell.

1 Spies, Tom D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *Southern Med. J.*, 1945, **38**, 707.

2 Clark, Guy W., *Am. J. Med. Sci.*, 1945, **209**, 520.

3 Stokstad, E. L. R., and Jukes, T. H., PROC. SOC EXP. BIOL. AND MED., 1946, **62**, 112.

TABLE I.

Substrate added to Tyrode solution*	Final conc., μg per ml	No. of reticulocytes per 1000 nucleated cells: Avg†	Range
None	—	7 (20)	3-8
Pteroylglutamic acid (folic acid)	4.9	8 (2)	8-8
	.05	8 (4)	6-9
	.0005	8 (2)	5-10
	.00005	7 (2)	7-7
Hexaglutamyl-pteroylglutamic acid (vitamin B_c conjugate)	1.0	8 (2)	7-9
	.1	7 (2)	7-7
	.01	6 (4)	5-8
Rat serum	—	13 (20)	9-34
Normal human serum	—	15 (4)	9-24
Human pernicious anemia serum‡	—	7 (1)	—
Human pernicious anemia serum‡ and liver extract	.005	13 (1)	—
Human pernicious anemia serum‡ and pteroylglutamic acid	.0025	8 (1)	—
Liver extract§	.1	11 (6)	6-18
	.01	11 (16)	9-18
	.001	13 (1)	—
	.0001	10 (2)	7-14
	.00001	6 (2)	6-6

* All substrates were dissolved in glucose-free Tyrode solution. In the cases where serum was added, 1 volume of serum was added to 3 volumes of glucose-free Tyrode solution.

† Numbers in parentheses indicate number of different experiments.

‡ Taken from case of pernicious anemia in relapse.

§ Liver extract powder provided by The Armour Laboratories. This extract (Lot No. 11285A) was used in the preparation of a 15 U.S.P. unit liver extract. 17 mg was equivalent to 1 U.S.P. unit.

send[4] in this and other laboratories likewise have failed. It would appear likely, therefore, that either the antipernicious anemia principle produced by the interaction of Castle's "extrinsic" and "intrinsic" factors, and that stored in the liver, are free of pteroylglutamic acid; or that pteroylglutamic acid is a component of the antipernicious anemia factor which ordinary methods of hydrolysis and assay fail to liberate and detect. The report of Welch *et al.*[5] that gastric juice did not liberate free pteroylglutamic acid from the heptaglutamyl (vitamin B_c Conjugate) form would support the latter view. From this report, there also appears to be some difference between the pernicious anemia patient and the normal individual with respect to the ability to break down the conjugated form to free pteroylglutamic acid. This finding agrees with the report of Bethel *et al.*[6] that patients having pernicious anemia fail to excrete increased amounts of pteroylglutamic acid in the urine when the heptaglutamyl conjugate form is fed. On the other hand, normal individuals are able to convert the conjugate form into pteroylglutamic acid and excrete it. In both instances pteroylglutamic acid when administered orally causes a marked increase in the urinary excretion of free pteroylglutamic acid.

In spite of the similarity between the clin-

[4] Castle, W. B., and Townsend, W. C., *Am. J. Med. Sci.*, 1929, **178**, 764.

[5] Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V., *J. Biol. Chem.*, 1946, **164**, 787.

[6] Bethell, F. H., Swendseid, M. E., Bird, D. D., Meyers, M. C., Andrews, G. A., and Brown, R. A., *Univ. Hosp. Bull., Univ. of Michigan*, 1946, **12**, 42.

ical response obtained from the administration of antipernicious anemia liver fractions and pteroylglutamic acid in the treatment of the various blood dyscrasias it is becoming apparent that the actual stage at which these 2 substances stimulate erythropoiesis may not be the same. In addition, it is known that on a weight for weight basis, liver extracts are more potent than pteroylglutamic acid or its heptaglutamyl form.

The work reported here is the result of a series of experiments that has been conducted in this laboratory during the past year comprising a study of the effect of pteroylglutamic acid,‡ its heptaglutamyl conjugate form,§ and liver fractions highly potent with respect to their antipernicious anemia content upon red cell maturation *in vitro* in an attempt to secure information concerning the point at which these substances act upon red cell production. After removing the immature cells from other marrow constituents by means of centrifugation the resulting preparation permits a study of the maturation process independent of the modifying factors of the gastro-intestinal tract, the liver, and the bone marrow matrix which are found in the intact animal and hence it is possible to study this one phase of red cell production separately.

Table I summarizes some of the results which were obtained by using bone marrow cell survival technic based upon the method of Osgood and Brownlee.[7,‖] Rat bone marrow cells were incubated 3-5 hours at 37°C while suspended in a glucose-free Tyrode solution. At the end of the incubation period, reticulocyte counts were made using the wet preparation technic and staining with brilliant cresyl blue. Only "mature" reticulocytes were counted. Lang-Levy micropipettes[8] were used in adding the various constituents to the media. A standard liver extract of known concentration was included in each day's experiment.

It will be noted that neither pteroylglutamic acid nor its heptaglutamyl conjugate, appeared to bring about maturation of red cells from their primitive precursors to the mature reticulocyte stage. On the other hand, a liver preparation produced maturation quantitatively similar to that change observed when normal serum is added to the preparation. It should be noted that in a single instance serum from a patient with pernicious anemia in relapse did not show the presence of this maturation factor. It is possible that this experiment may indicate that the stimulant for erythropoiesis found in the blood is similar to that found in liver extract and not similar to pteroylglutamic acid.

The difference between the effect of liver extracts and of pteroylglutamic acid upon immature bone marrow cells has been noted in the presence of glucose, casein hydrolysate, or glucose and casein hydrolysate in the same medium so that it appears not to be a function of a metabolite or a combination of amino acids present. It has been observed that ferrous iron added to any of the above substrates appears to diminish maturation.

Summary. The above evidence indicates that pteroylglutamic acid, or its conjugated form, does not act directly in an unaltered state upon primitive red cells to mature them, whereas a highly potent antipernicious anemia liver preparation appears to cause a maturation comparable to that observed in the presence of serum. A single sample of serum from a patient having pernicious anemia in relapse appeared to lack the maturation factor found in normal human and rat serum. Pteroylglutamic acid failed to increase the maturation of bone marrow cells suspended in this serum.

‡ Courtesy of Lederle Laboratories, Pearl River, N.Y.

§ Courtesy of Dr. J. J. Pfiffner, Parke, Davis and Co., Detroit, Mich.

7 Osgood, E. E., and Brownlee, Inez E., *J. A. M. A.*, 1936, **107**, 123.

‖ Details of this method are to be published.

8 Levy, M., *C. R. trav. Lab. Carlsberg, Serie chim.*, 1936, **21**, 101.

15673 P

Bacterial Variations in Salicin Medium.

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Most investigators have concerned themselves with the variation in morphological and colonial characteristics, especially in regard to the smooth (S) and rough (R) types. Neisser,[1] in his first work on variation, noted that a nonlactose-fermenting, colon-like organism gave daughter colonies which fermented lactose. Burri[2] observed this same phenomenon in regard to sucrose fermentation, when he studied an organism isolated from fermenting grass.

Many investigators have found no characteristic biochemical difference among their variants when the original culture was grown in media containing carbohydrates or higher alcohols except lactose.

Variation occurs spontaneously under ordinary conditions of cultivation, and also with environmental stimuli such as the presence of sugars, dyes, or changes in temperature. The presence of salicin in the medium as the environmental stimulus was chosen in this present investigation. The chief object was to study the characteristics of the variants which were produced when separate individual colonies from the mother culture were successively transplanted in salicin broth for 15 times.

A group of 36 salicin-negative organisms were selected, 31 of these belonging to the *Escherichia* genus and 5 to the *Aerobacter* genus. Eosin-methylene blue plates were streaked from these cultures, and 10 colonies which showed no gas production in salicin medium were transferred every third day to fresh tubes of salicin medium for at least 15 times. The percentages of gas produced were recorded after 7 days incubation at 37°C. As an example, a record of the acquired ability of culture *E. anindolica* G. to ferment salicin is shown in Table I.

Of the 36 organisms used, 22 (220 sub-cultures) remained negative to salicin throughout the 15 transplants in salicin broth. Of the other 14 organisms (140 sub-cultures), there were from 1 to 10 of the sub-cultures of each organism which acquired the ability to ferment salicin after from 1 to 12 transplants. Seventy-six of these 140 sub-cultures fermented salicin and 64 remained negative. Ten of the 64 salicin-negative sub-cultures were streaked on eosine methylene blue agar plates. Ten colonies were selected from each plate and placed in salicin medium. Positive variants were obtained from 7 of the salicin-negative daughter colonies. In no instance, however, did more than one tube out of 10 representing a given culture produce more than 10% gas. In many instances, it was discovered that the salicin-negative and salicin-positive variants from the same culture gave different forms of colonies on agar plates.

A representative number of the salicin-positive variants were placed on agar slants for permanent cultures. All of these cultures were transferred to salicin broth after being stored for 3 years at about 4°C. In every instance, where the organisms were still alive, the reaction to salicin was still positive. Streaks of 20 of the cultures were made on eosin-methylene blue agar. Ten of the individual colonies from each culture were grown in salicin medium for 7 days. The majority of the sub-cultures produced gas in salicin medium.

The quantitative study of the progressive acid fermentation was conducted on the original culture of *Aerobacter cloacae* and on one of its variants which developed the power to ferment salicin. These 2 organisms were grown in salicin medium, and at frequent intervals during 180 hours, the pH values of the medium were determined. Similar determinations were made for a culture of *Escherichia formica* and one of its salicin-

[1] Neisser, M., *Centralbl. f. Bakt.*, Abt. I, Ref., 1906, **38**, 98.

[2] Burri, R., *Centralbl. f. Bakt.*, Abt. II, 1910, **28**, 321.

ical response obtained from the administration of antipernicious anemia liver fractions and pteroylglutamic acid in the treatment of the various blood dyscrasias it is becoming apparent that the actual stage at which these 2 substances stimulate erythropoiesis may not be the same. In addition, it is known that on a weight for weight basis, liver extracts are more potent than pteroylglutamic acid or its heptaglutamyl form.

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Summary. The above evidence indicates that pteroylglutamic acid, or its conjugated form, does not act directly in an unaltered state upon primitive red cells to mature them, whereas a highly potent antipernicious anemia liver preparation appears to cause a maturation comparable to that observed in the presence of serum. A single sample of serum from a patient having pernicious anemia in relapse appeared to lack the maturation factor found in normal human and rat serum. Pteroylglutamic acid failed to increase the maturation of bone marrow cells suspended in this serum.

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7 Osgood, E. E., and Brownlee, Inez E., *J. A. M. A.*, 1936, **107**, 123.

‖ Details of this method are to be published.

8 Levy, M., *C. R. trav. Lab. Carlsberg, Serie chim.*, 1936, **21**, 101.

ammonium phosphomolybdate weighs 69 mg and the strychnine phosphomolybdate 89.3 mg; (2) the strychnine precipitate is more insoluble and forms much more rapidly; (3) the strychnine precipitate readily coagulates into large particles which can be more easily handled than the ammonium precipitate and have less tendency to adhere to the walls of the precipitating vessel; and (4) the strychnine precipitate can be dried to constant weight in an ordinary drying oven at 100° to 110°.

With the discovery that the solid polyethylene glycols form highly insoluble complexes with phosphomolybdic acid in the presence of barium,[2] the possibility suggested itself that these compounds might be adapted to a gravimetric determination of phosphate even more advantageously than strychnine. Subsequent experimentation has justified this expectation, and the present report describes the results obtained with the substitution of a high molecular weight polyglycol for strychnine in the Embden-Fetter method for the determination of inorganic phosphate in blood and urine. The barium-polyglycol-phosphomolybdate precipitate is the equal of strychnine phosphomolybdate on each of the last 3 counts mentioned above, and is superior in that it weighs 97.1 mg per mg of P. Its sole disadvantage is that its formation requires the presence of barium and, conversely, the absence of sulfate. This interference, however, may readily be eliminated by a precipitation preceding the filtration that accomplishes deproteinization of the sample. Minor considerations in favor of the use of the polyglycols are their low cost and availability since they are synthetic chemicals produced in tonnage capacity.

Reagents. All reagents employed, with the exception noted, are those specified in the Embden-Fetter method.[1] The polyglycol used is a commercial product sold under the trademark* name of "Carbowax" compound 6000, the numeral indicating a mixture of polymers with a mean molecular weight of 6000. As purchased, this material contains a small quantity of phosphate as an impurity, which may be removed by adsorption on basic ferric acetate in the following manner: Twenty g of the polyglycol are dissolved in *ca.* 160 ml of water and the solution is made faintly acid to methyl red by the addition of a few drops of dilute acetic acid. Then there are added in succession 4 ml of a 10% solution of ferric chloride in 0.2 N hydrochloric acid and 8 ml of a 5% solution of ammonium acetate. The mixture is heated with continual agitation until boiling just begins, at which point it is cooled momentarily and a few drops of dilute ammonium hydroxide are added. The voluminous precipitate which forms is immediately filtered. The filtrate, which is the polyglycol solution to be employed in subsequent work, should be water-white or of only a very faint greenish color, and should give no turbidity upon mixing with the molybdate solution. The precipitating reagent is prepared within an hour of use by mixing one volume of the polyglycol solution with three volumes of the molybdate reagent, in the same manner as the strychnine molybdate is prepared.

No sulfate was encountered in either of the two samples of "Carbowax" compound 6000 used in this work; however, had this anion been present, it would obviously have been necessary to have removed it prior to employment of the reagent. This removal could probably be best effected by acidifying the original polyglycol solution, adding barium chloride, and filtering with suction through fine asbestos. The reaction of the filtrate would then be adjusted with dilute ammonium hydroxide, and the removal of phosphate carried out from that point.

Procedures. (A) For Blood. Five ml of blood, plasma, or serum are measured into a 50 ml flask containing 25 ml of 10% trichloracetic acid. Following a thorough shaking of the coagulum, 5 ml of a 10% barium chloride solution are added, and the flask is filled to the mark with water. After about 15 minutes of standing, the contents are filtered through Whatman No. 42 or comparable paper. Twenty-five ml of the filtrate are diluted to 60 ml, and 20 ml of the polyglycol-molybdate solution are added. The mixture is allowed to stand at room temperature for one hour

[2] Shaffer, C. B., and Critchfield, F. H., *Ind. Eng. Chem., Anal. Ed.*, 1947, in press.

* Carbide and Carbon Chemicals Corporation.

TABLE I.
Production of Gas in Salicin Medium of Variants of *E. anindolica* G.

No. of transplant	Daughter colonies									
	1	2	3	4	5	6	7	8	9	10
1	0*	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	5	10	0	20	10	0	0	0	0	5
7	10	10	5	25	15	0	0	0	0	15
8	10	10	5	25	30	0	0	0	20	10
9	20	20	15	25	25	0	20	0	25	20
10	20	25	25	20	15	5	15	0	20	15
11	20	25	35	20	15	20	25	0	20	30
12	30	20	30	20	15	20	20	20	20	35
13	35	35	30	25	15	20	20	30	20	40
14	35	35	20	25	15	30	25	35	20	40
15	35	35	30	30	25	30	30	35	35	40

* Figures are in percentages of gas produced in 7 days.

positive variants.

It was discovered that the salicin-positive variants developed a greater capacity to form acid from salicin. The original cultures (salicin-negative) showed an acid production for about 10 hours to a minimum pH of about 6.7, after which there was an abrupt rise in pH to over 8.5 during 180 hours. The salicin-positive variants from these cultures reduced the pH value of the medium to a value around 5 in 10 to 30 hours. The acid production was so great that the organisms were killed in most instances.

The results of this investigation indicate that, the fermentation of a given substance, such as salicin, is not necessarily a constant characteristic. Because of the acquired ability to ferment this glucoside, a number of organisms changed from one species to another according to the Bergey classification. Five cultures change their classification from *E. anindolica* to *E. communior*, 3 from *E. formica* to *E. coli*, and 3 from *E. gruenthali* to *E. paragruenthali*.

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High Molecular Weight Polyglycols as Reagents in the Gravimetric Determination of Inorganic Phosphate.

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Despite the availability of the simpler, direct colorimetric methods for the determination of small amounts of inorganic phosphate in biological materials, gravimetric methods, by reason of their greater precision and accuracy, have continued to find some application. Two precipitates that have been employed for quantities of phosphate too small for the magnesium ammonium phosphate method are the ammonium and strychnine salts of phosphomolybdic acid. The strychnine phosphomolybdate appears to have gained wider preference; its advantages over the ammonium salts have been summarized by Peters and Van Slyke[1] as follows: (1) the weight of the precipitate is greater, for per 1 mg of P the

[1] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Vol. II, Methods, pp. 849, 873, Baltimore, The Williams and Wilkins Co., 1932.

as 0.0103.

Results. Table I lists the results of inorganic phosphate determinations of 13 different samples of dog plasma and 13 different samples of human urine carried out by the proposed method, as compared with duplicate determinations performed simultaneously on the same specimens by the Embden-Fetter strychnine-phosphomolybdate method. Statistical treatment of these values by the application of the t distribution indicated no significant difference between the results obtained.

In the analysis of whole blood, correlation of the results obtained by the two methods on a given sample is good provided that filtration of the precipitate is performed promptly at the conclusion of the one-hour period of standing recommended. However, when this time of standing is prolonged, hydrolysis of organic phosphate appears to take place more extensively in the strychnine-precipitated sample and to make the results from this procedure high as compared with those from the other.

Summary. A method is described for the precipitation of phosphate phosphorus as a barium-polyglycol-phosphomolybdate complex which may be filtered and dried to constant weight at 100°-110°. Evidence is presented to show the applicability of this reaction to phosphate determinations on biological materials, and a comparison is made of this procedure with the strychnine phosphomolybdate (Embden-Fetter) method.

15675

Effects of Vital Dyes on Early Development of the Amphibian Embryo.

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A vital dye may be used to stain different cells and regions of eggs and embryos. To be successfully employed in this fashion, the dye should be "non-toxic and harmless so as not to destroy or even impair the vital activities and embryonic performance of its carrier" (Weiss[1]). However, it has been shown by Child and his co-workers,[2] in the work on the occurrence of physiological gradients, that so-called "vital" dyes, especially the basic ones, can be quite toxic. Determination of the conditions under which vital staining of eggs and embryos can be successfully accomplished is of interest. The general effects of two basic dyes, Nile Blue Sulfate and Neutral Red, were studied by Detwiler[3] in Amblystoma embryos. Gersch[4] and Gersh and Ries[5] in their work on sea-urchin eggs, found that different concentrations of the acid dye, Trypan Blue, caused severe developmental anomalies. Inhibition of mitosis was shown to occur in the larvæ of *Triton taeniatus* after a one- to two-hour exposure to certain concentrations of Neutral Red (Luther[6]). However, Uschin[7] who studied the same problem in *R. temporaria* tadpoles found no dye effects on mitosis.

In view of the frequency with which the

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1 Weiss, P., *Principles of Development*, 1939, Henry Holt and Co., New York.

2 Child, C. M., *Patterns and Problems of Development*, 1941, The University of Chicago Press.

3 Detwiler, S. R., *Anat. Rec.*, 1917, **13**, 493.

4 Gersch, M., *Arch. Entw. Mech.*, 1937, **136**, 210.

5 Gersch, M., and Ries, E., *Arch. Entw. Mech.*, 1937, **137**, 169.

6 Luther, W., *Klin. Woch.*, 1939, **18**, 682.

7 Uschin, N. P., *Arch. f. Exp. Zellforsch.*, 1931, **11**, 472.

TABLE I.
Comparison of Phosphate Determinations on Random Samples of Plasma and Urine Performed by the Reference Method and Duplicated by the Proposed Method.

	Phosphate P per 100 ml			
	Plasma		Urine	
Sample No.	Strychnine mg	Polyglycol mg	Strychnine mg	Polyglycol mg
1	5.15	5.36	79.14	80.55
2	3.72	3.72	115.12	117.52
3	4.37	4.15	4.07	4.02
4	3.52	3.44	69.41	68.91
5	4.44	4.64	27.42	28.02
6	3.52	3.52	56.49	57.06
7	3.56	3.52	169.83	172.21
8	2.82	3.08	32.08	32.14
9	3.78	3.60	70.71	71.07
10	2.90	2.84	109.67	109.18
11	3.38	3.52	29.85	30.38
12	2.90	3.04	77.26	79.00
13	4.68	4.92	86.02	85.49
Mean	3.75	3.80	71.31	71.96

with occasional shaking to complete the precipitation. The precipitate is then filtered in a small Gooch crucible previously dried at 110°, cooled in a desiccator, and tared. It is important to use only gentle suction during filtration. Particles of the precipitate which adhere to the container are rinsed into the crucible with 25 ml of ice-cold, diluted (1:5) molybdate solution. The precipitate is then washed with ice-cold water until the washings are no longer acid. During the washing, as soon as one portion of fluid has been drawn through the crucible another portion is added or else the suction is stopped, to prevent the precipitate from becoming divided by cracks through which subsequent portions of wash water can run with little contact with the precipitate. The crucible is dried for an hour at 100°-110°, cooled in a desiccator, and weighed.

(B) For Urine. Five ml of urine, well mixed to suspend any insoluble phosphates, are measured into a 50 ml volumetric flask together with 10 ml of water and 25 ml of 10% trichloracetic acid. Five ml of a 10% solution of barium chloride are then added dropwise, and the flask is allowed to stand undisturbed for one-half hour. At the end of this time the contents are shaken and made up to the mark with water. The barium sulfate precipitate, together with any coagulum in the flask, is separated by filtration, or by centrifugation if necessary, and an aliquot of the clear filtrate (usually 10 to 20 ml depending upon phosphate content) is diluted to 60 ml with water, 5 ml more of the barium chloride solution are added, and the rest of the determination is carried out as described above for blood.

(C) Determination of gravimetric factor. Just as in the case of the strychnine precipitate, each analyst should determine for himself the factor indicating the mg of P per mg of barium-polyglycol-phosphomolybdate precipitate. It has been found that different preparations of strychnine may give different weights of precipitate per mg of P, and it may be assumed that less homogeneous polyglycols will show some limited variation in this respect, although samples from 2 different production batches tested in this work gave identical results. For determination of this factor, a standard phosphate solution containing 0.4395 g/l of thoroughly dried reagent grade KH_2PO_4 was prepared. Aliquots containing 0.5 to 1.5 mg of P (5 ml to 15 ml of solution) were measured from a certified buret, diluted to 60 ml with water, 5 ml of barium chloride solution were added, and the phosphate was precipitated and weighed in the manner described. From a series of 15 trials the gravimetric factor was calculated

TABLE II.
Fifteen Hours Exposure to Nile Blue Sulfate.

Hr after fertilization when immersed	Stage No. and description at time of immersion Control and experimental	Description at time of removal. Control and exper.	% showing any abnormality		% surviving through St. 25	
			1:150,000	1:100,000	1:150,000	1:100,000
1	2—grey crescent	mid-cleavage	0	29	97	71
6	6—16 cells	" "	0	25	98	81
22	9—late cleavage	mid-gastrula	48	84	62	33
26½	10—dorsal lip	late "	17	79	78	28
39	11—mid-gastrula	early neural plate	1	80	98	75
45½	12—late gastrula	" " folds	0	76	98	72
63	14—neural folds	neural tube	0	3	100	97

the dye accumulating within the cells reached a concentration incompatible with normal development, eggs immersed at the 2-cell stage, were allowed to remain for graded lengths of time, 3, 6, 12, 18, 24, 36, 48, 67 hours, and then were transferred to fresh spring water for further growth and development. A summary of the maximum exposure to different concentrations which did not interfere with normal development is given in Table I.

A result which might be due to a delayed effect of exposure to the dyes was observed in this series. Eggs exposed for short periods of time to the stronger concentrations developed normally while in the dye solutions and also for some time after removal to fresh medium, but then suddenly became abnormal at the time of gastrulation. For example, eggs which were exposed to 1:50,000 Nile Blue Sulfate from the 2-cell stage to a many-cell stage (a 6-hour exposure) developed normally through this period and for approximately 18 additional hours in fresh spring water. At the end of these 18 hours the eggs reached the dorsal lip stage and the abnormalities already described began to manifest themselves.

III. Effect of stage of development on results of exposure to dyes. Since the first observations indicated a great sensitivity of gastrular stages, a separate study was made of the influence of developmental stage on the results. Eggs were exposed to different concentrations of Nile Blue Sulfate for 15 hours while at different stages of development. In Table II are given the number of hours after fertilization and a description of the development at the time of exposure and removal from the dye solutions.

Of all the concentrations used, 1:100,000 and 1:150,000 were found to be critical for a demonstration of the phase susceptibility (Table II, columns 4, 5, 6, 7). Exposure starting at least one hour before the first cleavage did not delay the onset of division or affect the rate or character of the following divisions. Any departures from the normal occurred at gastrulation, long after removal from the dye. The results were similar for eggs exposed at 6 hours after fertilization (st. 6). Starting at st. 9 an increase in the sensitivity of the eggs was noted as indicated by a rise in the percentage of eggs showing abnormalities as compared with the st. 2 and st. 6 eggs. In a concentration of 1:150,000, the percentage of cases of abnormality decreased in stages 11 and 12. In the higher concentrations of 1:100,000 where it is quite likely that the rate of penetration of dye per unit time was higher, the percentage of abnormality remained large. Nevertheless, the decrease in susceptibility was demonstrable, since the abnormalities produced were less severe and a higher percentage of survival resulted. Of the eggs exposed at st. 11 and st. 12, 75 and 72% respectively were able to survive to st. 25 (the endpoint of the experiment) while of the eggs exposed at st. 9 and st. 10 to the same concentration for the same length of time only 33 and 28% respectively reached the same point. No effect on development was noted when exposure was made at st. 14.

Thus it would seem that in the development of eggs through the neurula stage, late cleavage and dorsal lip stage (st. 9 and st. 10), were the most sensitive to the presence of dye since exposure at these times produced

eggs of the leopard frog, *Rana pipiens,* are used in experimental embryology studies, an investigation of their response to exposure to vital dyes under different conditions seemed of practical importance.

Materials and Methods. The dyes used were Nile Blue Sulfate, Neutral Red and Bismark Brown. Fresh stock solutions containing 100 mg of commercial dye† in 1000 cc of solvent (spring water‡) were prepared before each experiment and diluted as required. Eggs of *R. pipiens* (Vermont stock) were obtained in the laboratory by the method of induced ovulation and fertilization described by Rugh[8] and were allowed to develop in finger bowls containing 200 cc of solution; between 20 and 30 eggs per bowl. A total of 2050 eggs were used. Throughout the course of the experiments, the eggs were studied for both character and rate of development. This was recorded in terms of stages of normal development of *R. pipiens* as described by Shumway.[9]

Observations. I. Concentration. Eggs were placed in dye solutions of different concentrations ranging from 1:10,000 to 1:1,000,000 while in the 2-cell stage (st. 3) and were allowed to develop therein up to the complete operculum stage (st. 25). For each dye it was possible to determine a "critical" concentration below which development was completely normal and above which abnormalities and lethal effects began to appear. This critical point varied with each dye, being lowest with Nile Blue Sulfate (1:750,000), considerably higher with Neutral Red (1:250,000) and just slightly higher with Bismark Brown (1:100,000).

Developmental abnormalities produced by exposure to concentrations above the critical levels were of the same nature for each of the dyes. A direct correlation between concentration and time of appearance of the abnormalities seemed to exist; the stronger the concentration the sooner development became abnormal, up to a certain point. However, even with the strongest concentrations used, 1:10,000 (below this point the eggs became so heavily stained that it was impossible to see the cleavage furrows) cleavage was always normal in both character and rate.

The gastrulation stages, beginning with the appearance of the dorsal lip of the blastopore (st. 10) were the first stages in which an effect on development could be noted. In many instances development stopped entirely immediately after the formation of the dorsal lip; in others, development continued but in an abnormal fashion. The effect seemed to be one which involved an interference with the growth of the cells of the animal hemisphere over the large yolk-laden cells of the vegetal hemisphere. Consequently masses of yolk material remained exposed.

All embryos with exposed yolk material which survived beyond gastrulation showed imperfect neurulation. This consisted of an interference with either the formation or the closure of the neural folds. In most cases neural folds could form in the anterior region, and therefore the state of development found in that region was used as a basis for the classification of the embryos into stages. All degrees of abnormalities were found from the cases in which the entire dorsal region of the embryo was occupied by yolk material to those in which only a small yolk plug blocked the closure of the neural folds in the posterior region.

II. Timed exposure to vital dyes. In order to determine, for each concentration, when

TABLE I.

For each concentration is given the length of exposure in hours which did not interfere with normal development. Eggs exposed at stage 3 and maintained at 19 ± 1.5°C. (220 hours represent the time needed to reach st. 25, the end point of these experiments).

Concentration × 1000	Nile blue sulfate	Neutral red	Bismark brown
1:25	—	<3	90
1:50	<3	6	150
1:75	—	12	—
1:100	12	72	175
1:250	24	220	220
1:500	110	220	220
1:750	175	220	220
1:1,000	220	220	220

† Obtained from the National Aniline and Chemical Company, N.Y.

‡ Purchased from the Great Bear Spring Company, N.Y.

8 Rugh, R., *Experimental Embryology, A Manual of Techniques and Procedures,* 1942, New York University Bookstore, New York, N.Y.

9 Shumway, W., *Anat. Rec.,* 1940, **78,** 139.

6. The pH of the medium was shown to influence the toxicity effects.

7. When Nile Blue Sulfate, Neutral Red and Bismark Brown are used as vital dyes, the factors of concentration, length of exposure and stage of development must be considered to avoid induced abnormalities in development, most of which begin at the time of gastrulation.

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A Method for Producing Experimental Venous Thrombosis.*

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In our attempts to study the genesis and functional pathology of intravascular thrombosis, and to determine the *in vivo* action of heparin on preformed clot, it was found necessary to devise a method of experimental clot formation in animals which produced consistent, predictable results. This report deals briefly with such a method successfully employed in rabbits.

Experimental venous thrombosis has hitherto been accomplished by chemical and mechanical means.[1-4] The introduction of an extraneous factor in chemically-induced thrombosis unnecessarily complicates the proper evaluation of the effect of heparin. Accordingly, mechanical methods of inducing thrombosis are preferable. Vein crushing over an intraluminal silk thread,[5] although reported to be 80% successful, did not give us consistently good results. Similarly, we found stretching of the vein to induce intimal damage[6] successful in only 20% of instances.

The elaboration of a thrombus depends on (a) stagnation of blood, (b) injury to the intima, (c) local release of considerable amounts of thrombokinase. A method utilizing these 3 factors is herewith described. Three kilogram adult rabbits are anesthetized with ether and a midline cervical incision is made. The jugular veins on either side are exposed. Both veins are treated alike. A 3-cm segment of vein is dissected free and the most proximal portion securely tied with a silk ligature. A flat, narrow strip of metal, such as a ribbon retractor, is placed under the vein distal to the ligature and acts as an anvil. The vein is then given 15 to 30 sharp taps with the handle of a Mayo scissors. Brisk bleeding will occur which is readily controlled by gauze pressure. Care is taken not to fracture the vein completely across. When bleeding has ceased, usually in about 2 minutes, a palpable and visible thrombus appears. If clotting does not occur, the procedure is again repeated. Clotting invariably is present after the second series of strokes. All animals are reexamined after 48 hours to reaffirm the presence of clots. Examination and study of these clots, in the gross and in microscopic sec-

* Aided by a grant from the William R. Warner & Co., Inc., New York.

1 Zahn, F. W., *Virchow's Arch. f. path. Anat.*, 1875, **62**, 81; *ibid.*, 1884, **96**, 1.

2 Eberth, J. C., and Schimmelbusch, C., *Virchow's Arch. f. path. Anat.*, 1886, **103**, 39; *ibid.*, 1886, **105**, 456.

3 Welch, W. H., *Papers and Addresses*, Vol. 1, p. 110, Baltimore, 1920, Johns Hopkins Press.

4 Zurhelle, E., *Zeigler's Beitr. z. path. Anat. u. z. allg. Path.*, 1910, **47**, 539.

5 Murray, D. W. G., Jaques, L. B., Perrett, T. S., and Best, C. H., *Surgery*, 1937, **2**, 163.

6 Rabinovitch, J., and Pines, B., *Surgery*, 1943, **14**, 669.

the largest number of abnormalities and the poorest survival. The question is raised as to whether the high sensitivity seen at st. 9 might not have been due to the fact that in the 15-hour period of exposure, the eggs developed through st. 10 while still in the dye.

IV. Effect of hydrogen ion concentration. The pH of the dye solutions used was found to vary from pH 6.8-6.9 at the time of preparation and to change within a period of 24 hours to pH 7.3-7.4 (probably due to the alkalinity of the glassware used). Therefore, dye solutions buffered to different pHs from pH 4.5 to pH 8.5 were prepared and the staining results compared with those of the unbuffered solutions. It was found that even at high concentrations development proceeded normally through st. 25 in the most acid solutions and that the larvae were only very faintly stained even after long periods. At pH 6.3 to 8.5 the effects were the same as have been described for the unbuffered solutions and also the larvae were all very heavily stained. This agrees substantially with the observations of MacArthur[10] on the staining of *Planaria dorotocephala* with basic dyes and also with the work of Chambers, Irwin and others[11] on the effects of pH on the penetration of dyes into unicellular forms. The phenomenon can be explained either on the basis of the dissociation of the dyes or on the basis of the effect of pH on the particle size (Gordon & Chambers).[12]

Discussion. It has been shown that unless the concentration used, the length of exposure, the stage of development of the eggs, and the hydrogen ion concentration are carefully considered, exposure of the eggs of *R. pipiens* to solutions of vital dyes, Nile Blue Sulfate, Neutral Red and Bismark Brown, may result in abnormal development. This is especially true when the early cleavage stages are used because although cleavage itself remains unaffected the stages which follow are severely damaged.

Nile Blue Sulfate produces good staining but must be used in very low concentrations. Neutral Red is considerably less toxic and Bismark Brown is even less so. As far as could be determined here, there is no difference in the affinity of the 3 dyes for the eggs used. All 3 can readily be seen in fixed sections and also in the living state even though the eggs of *R. pipiens* contain a large amount of dark pigment which has a tendency to mask the color somewhat. These facts would seem to indicate the advisability of using Neutral Red and Bismark Brown in experiments which involve the staining of entire eggs and embryos. The best staining results are obtained by long exposures to weak concentrations rather than by short exposures to strong concentrations. Better results are also produced if the eggs are exposed to the dyes without removal of the jelly capsule. This was also noted by Detwiler[3] for the eggs of Amblystoma. The color produced is uniform throughout the eggs, and the ectoderm is perfectly normal.

Summary. 1. Eggs of *Rana pipiens* were exposed to solutions of the vital dyes Nile Blue Sulfate, Neutral Red and Bismark Brown under different conditions. Concentrations less than the following allowed normal development of eggs and embryos: Nile Blue Sulfate 1:750,000; Neutral Red 1:250,000; Bismark Brown 1:100,000.

2. Developmental abnormalities appeared with use of stronger concentrations than listed above.

3. Early cleavage was unaffected by otherwise toxic concentrations even to 1:10,000. Gastrulation proved to be the critical point in development when toxic effects of the dyes began to produce abnormalities.

4. Short exposure to toxic concentrations, even during unaffected cleavage, resulted in abnormalities appearing at subsequent stages of development.

5. Developmental stages *9* and *10* were more susceptible to toxic effects than were stages *2, 6, 11, 12, 14* using Nile Blue Sulfate of 1:100,000 and 1:150,000 concentrations.

10 MacArthur, J. W., *Am. J. Physiol.*, 1921, **57**, 350.

11 See review of Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes*, 1943, Cambridge University Press.

12 Gordon, H. K., and Chambers, R., *J. Cell. Comp. Physiol.*, 1941, **17**, 97.

Fig. 4. Microscopic section of Fig. 3 (30 ×) showing organization and recanalization.

Fig. 3. Jugular veins of rabbit 14 days after ligation and hammering. The veins are thickened and fibrotic.

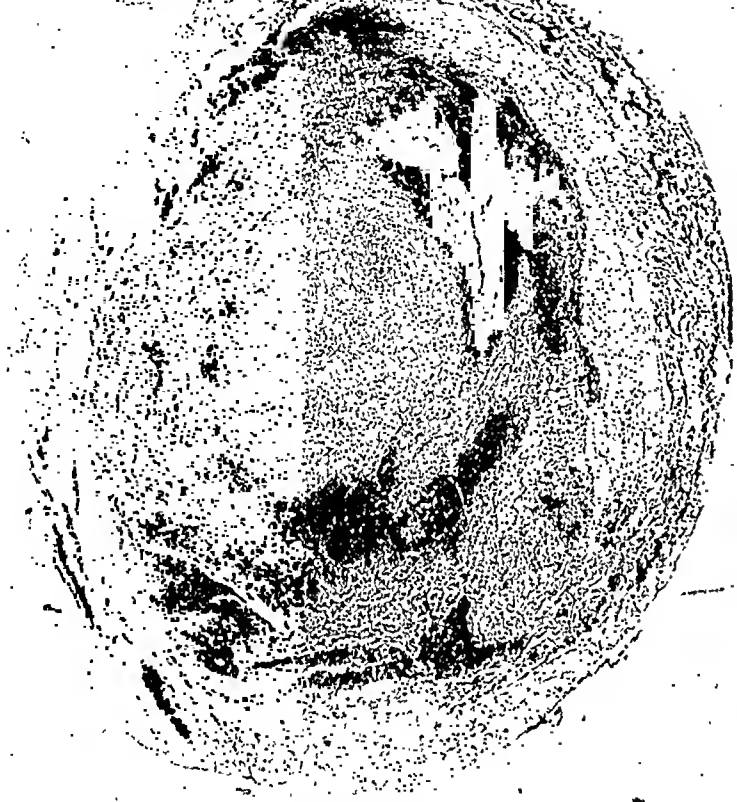

FIG. 2.
Microscopic section of Fig. 1 (38 ×) showing organization of clot and beginning recanalization.

FIG. 1.
Jugular veins of rabbit 6 days after ligation and hammering.

Fig. 4. Microscopic section of Fig. 3 (30 ×) showing organization and recanalization.

FIG. 3. Jugular veins of rabbit 14 days after ligation and hammering. The veins are thickened and fibrotic.

tion,† reveals that they differ in no way from the spontaneous *in vivo* clot seen in aseptic thrombophlebitis. The progressive morphologic changes leading to organization and recanalization are also similar to spontaneous *in vivo* clots (Fig. 1, 2, 3, 4). In the last 140 veins, the procedure has been successful in every case.

Conclusion. An easily controlled, readily available method of experimental clot production is described. It has been uniformly successful in our hands.

† We wish to thank Dr. David M. Grayzel for his interpretation of the microscopic sections.

15677

Viscosity of Normal Human Synovial Fluid.

CHARLES RAGAN. (Introduced by W. W. Palmer.)
(With technical assistance of Audrey F. Grubin.)

From the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital and the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York.

Synovial fluid is a viscous solution containing a longchain mucopolysaccharide (hyaluronic acid), protein and electrolytes.[1] The relative viscosity of such fluid is related to the amount of hyaluronic acid present[2-4] and to the state of polymerization and aggregation of the polysaccharide.[5] When joint fluid is incubated with an hyaluronidase,[6] the viscosity approaches but does not quite reach the viscosity of water.[6] The slight residual increase in viscosity may be due to the proteins present which are unaffected by purified preparations of hyaluronidase. (Isolated pure hyaluronic acid never attains the high viscosity found in native (human or animal) synovial fluids, presumably because the procedures used in isolation disaggregate the polysaccharide as it occurs in the native form.[5] The human knee joint is functionally far different from the 4-legged animal's astragalo-tibial joint from which most specimens of so-called normal joint fluid have been obtained.[3,7] It seemed of interest to determine, if possible, the characteristics of normal human synovial fluid, to compare with pathological fluids which can readily be obtained in large quantity. Reports on the viscosity of normal human synovial fluid have yielded wide variations and the technics of viscosimetry are open to some criticism.[8,9] When an apparently normal human knee joint is tapped at the postmortem table, one can rarely withdraw more than 1 or 2 cc of joint fluid. Thus, 2 to 4 cc of human knee joint synovial fluid can be obtained from the 2 knees of an individual, which is less than the capacity of the standard 5 cc Ostwald viscosimeter. On casual inspection, it was found that the fluid obtained was very viscous, and it was probable that a viscosimeter of less than 5 cc capacity would give an inaccurate reading with such viscous material.

Methods. Viscosities were measured in 5 cc Ostwald viscosimeters in a water bath at temperature of 21° ± 1°C. Total proteins were determined with a gradient tube method.[10] In this laboratory, it has been

1 Hesselvik, L., *Acta Med. Scand.*, 1940, **105**, 153.

2 Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114**, 689.

3 Meyer, K., Smyth, E. M., and Dawson, M. H., *J. Biol. Chem.*, 1939, **128**, 319.

4 Meyer, K., and Chaffee, E., *J. Biol. Chem.*, 1940, **133**, 83.

5 Meyer, K., personal communication.

6 Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

7 Ropes, M. W., Bennett, G. A., and Bauer, W., *J. Clin. Invest.*, 1939, **18**, 351.

8 Kling, D. H., *Arch. Surg.*, 1931, **23**, 543.

9 Schneider, J., *Biochem. Z.*, 1925, **160**, 325.

10 Lowry, O. H., and Hunter, T. H., *J. Biol. Chem.*, 1945, **159**, 465.

found that the gradient method has a good correlation with the Howe method in estimation of protein content of synovial fluid. It has also been shown that the specific gravity of a 1% solution of purified sodium hyaluronate* is below the scale of the gradient method employed and does not materially affect specific gravity estimation of synovial fluid protein.

Eight pathological joint fluids of varying viscosity were diluted with 0.9% sodium chloride, the relative viscosity at each dilution was plotted against dilution and curves were drawn (Fig. 1). It can be seen that the fluids with a very high initial viscosity show an abrupt fall on minimal dilution with

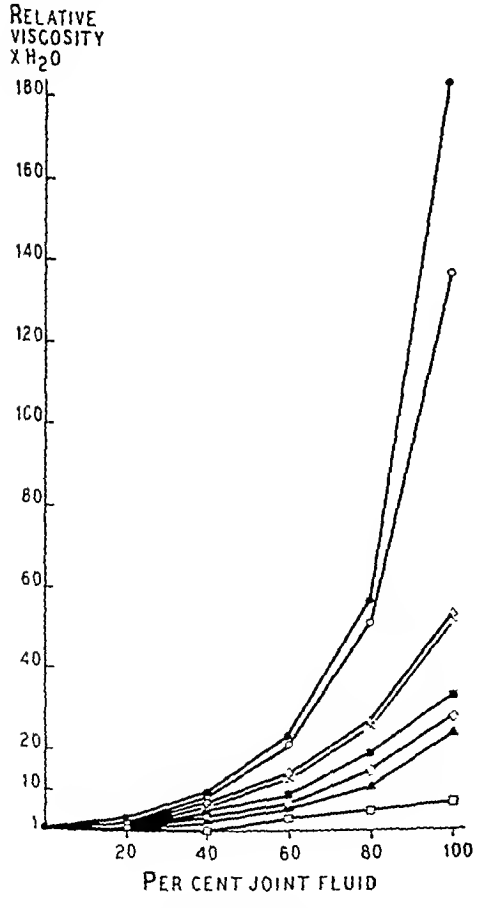

FIG. 1.
Curves of relative viscosity obtained by dilution of 8 pathological joint fluids with 0.9% NaCl.

* Obtained through the courtesy of Dr. K. Meyer.

TABLE I.
The Relative Viscosity and Total Protein Content of Normal Joint Fluids.

Pt.	Diagnosis	Peripheral oedema	Amt removed both knees, cc	Relative viscosity: Dilution, %	Rel. visc. diluted sample	Relative visc. by extrapolation	Total protein
C.H.	Carcinoma vocal cords	0	2	40	11.8	>185	—
F.B.	Hypertensive cardiovascular disease	0	2.5	50	36	>185	1.3
R.D.	Lymphosarcoma	0	3	40	8.9	134-184	—
S.McC.	Hypertensive cardiovascular disease, mesenteric thrombosis	0	3	50	88.2	>185	1.5
E.T.	Hypertensive cardiovascular disease	0	3.6	50	16.8	185	2.3
E.C.	Intestinal obstruction	0	3.6	50	12.6	60-134	2.4
H.H.	Carcinoma stomach	0	4	50	12.8	60-134	1.8
F.L.	Coronary occlusion	0	1	20	4.4	>185	—
T.W.	Lung abscess with terminal hemorrhage	0	2	40	10.1	>185	2.0
C.N.	Hypertensive cardiovascular disease	+++	rt. 30	100	—	3.0	1.6
			lt. 20	100	—	7.2	1.7
T.J.	Hypertensive cardiovascular disease	++	rt. 15	100	—	47.0	2.2
			lt. 12	100	—	62.2	2.2
N.S.	Nephrosis	++++	not all removed	50	5.1	7-20	.94

tion,† reveals that they differ in no way from the spontaneous *in vivo* clot seen in aseptic thrombophlebitis. The progressive morphologic changes leading to organization and recanalization are also similar to spontaneous *in vivo* clots (Fig. 1, 2, 3, 4). In the last 140 veins, the procedure has been successful in every case.

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7 Ropes, M. W., Bennett, G. A., and Bauer, W., *J. Clin. Invest.*, 1939, **18**, 351.
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9 Schneider, J., *Biochem. Z.*, 1925, **160**, 325.
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due to one of two factors or to both, (a) dilution with extracellular water, (b) depolymerization or dissociation of a highly polymerized hyaluronic acid by hyaluronidase.

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Inactivation of Poliomyelitis Virus in Relation to Gastric and Intestinal Digestion.*

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From the Department of Pediatrics, Stanford University School of Medicine, San Francisco, Calif.

Whether or to what extent gastric and intestinal digestion inactivates poliomyelitis virus is a problem that has an important bearing on intestinal entry of infection and on the source of virus in the intestinal contents and stools. This problem has not been studied as closely as might be desired in its relation to the conditions of normal digestion, although such studies as are available indicate that the virus displays a considerable degree of resistance to inactivation in the gastrointestinal tract. Thus, Flexner, Clark and Dochez[1] found active virus in the intestine 2 hours after administration by stomach tube; and by the same method of administration Clark, Schindler and Preston;[2] Clark, Roberts and Preston;[3] and Levaditi, Kling and Lepine[4] found active virus in the stools within 48 hours. The effects of H-ion concentration upon virus in the range with which we are here concerned has been studied by Loring and Schwerdt[5] but their experiments were limited to periods of 1-2 hours and were done at room temperature. So far as we know no studies on the inactivating effects of individual digestive enzymes on poliomyelitis virus have been recorded.

In the present experiments the test conditions were intended to approximate those obtaining in the human stomach in respect to temperature, time, pH range, and presence of pepsin. In children over one year of age (the group most susceptible to poliomyelitis), Cutter[6] has shown that the fasting gastric contents 10 minutes after stimulation with histamine display an average acidity of pH 1.5 and occasionally reach pH 1.2. In young adults, the level is pH 1.0-1.2.[7] Hammon and Izumi[8] showed that poliomyelitis virus is not inactivated at levels of pH 4.0 and up. Gastric evacuation begins a few minutes after ingestion.[9] At 4 hours, after ingestion of various types of food, the stomach is nearly empty.[9] On the basis of such data, we have selected for our studies a range of pH 1.0-4.0 and exposure periods of 5 minutes to 4 hours. All observations were made at 37°C, or approximate body temperature.

The Lansing (Armstrong) mouse-adapted strain of poliomyelitis virus was used. Stock 10% and 20% suspensions of infected mouse cord in physiological NaCl solution were clarified at 18,000 r.p.m. for ½ hour, then

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Flexner, S., Clark, P. F., and Dochez, A. R., *J. Am. Med. Assn.*, 1912, **59**, 273.

2 Clark, P. F., Schindler, J., and Roberts, D. J., *J. Bact.*, 1930, **20**, 213.

3 Clark, P. F., Roberts, D. J., and Preston, W. S., Jr., *J. Prev. Med.*, 1932, **6**, 47.

4 Levaditi, C., Kling, C., and Lepine, P., *Bull. Acad. Med.*, 1931, s. 3, **105**, 190.

5 Loring, H. S., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 173.

6 Cutter, R. D., *J. Pediat.*, 1938, **12**, 1.

7 Helmer, O. M., and Fonts, P. J., *Am. J. Clin. Path.*, 1937, **7**, Tech. Suppl., 41.

8 Hammon, W. D., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 579.

9 Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, 3rd Ed., 1943, Baltimore, The Williams and Wilkins Co.

TABLE II.
The Relative Viscosity and Total Protein Content of Pathological Joint Fluids.

Pt.	Diagnosis	Knee joint	Amt removed cc	Relative viscosity	Total protein
R.R	Rheumatoid arthritis—old	right	300	175	4.8
E. O'T.	" " —early	"	20	6.8	4.2
L.S.	Torn medial meniscus	left	15	13.8	4.6
H.T.	Rheumatoid arthritis—old	right	60	48.2	5.3
H.F.	" " "	"	40	16.8	4.8
P.R.	" " —early	"	20	5.6	5.9
T.B.	Gonococcal arthritis	"	20	8.4	4.4
A.N.	Arthritis one knee assoc. with lymphogranuloma inguinale	"	75	40.5	4.4
G.N.	Intermittent hydrarthrosis	left	40	11.2	5.9
M.P.	Gonococcal arthritis—old	"	40	8.8	6.0

an elliptical type of curve. This type curve may be superimposed on the dilution curve for pleural fluid reported by Meyer.[4]

It has been possible to approximate the viscosity of normal joint fluid by dilution and extrapolation on these curves This has been done by dilution with 0.9% NaCl and extrapolation on the curves in Fig. 1 (Table I). In normal joints, one factor which is very important is the amount of fluid which can be obtained at tapping. In this small series this seems to be directly related to the amount of peripheral edema present and thus the joint space can be looked upon, as Bauer[11] has felt, as an extracellular space.† Where no more than 5 cc was obtained on tapping both knees, the viscosity of the joint fluid was greater than or equal to the viscosity of our most viscous pathological joint fluid. In 2 instances, with rightsided heart failure and marked pretibial edema, but with apparently normal knee joints, a larger amount was withdrawn from the knees (Pt. C.N. and T.J. in Table I), and the viscosity of these was less than that seen in normal fluids. One patient on the ward with generalized anasarca in the nephrotic stage of chronic glomerulonephritis was tapped and 2.5 cc of fluid was removed from his right knee. This was not viscous and on dilution and extrapolation gave a viscosity of 7 to 20 times water. As shown in Table I, in the absence of peripheral edema, the calculated viscosity of normal joint fluid was very great compared to the viscosity of the joint fluid obtained from a random selection of pathological joints shown in Table II. The protein content of fluid from normal joints as measured by a specific gravity method was below 3.0%, while in pathological joints, the protein content of the fluid was above 3.0%. (Table I and Table II).

Discussion. The viscosity of synovial fluid from knee joints of patients without symptoms or signs of joint disease is equal to or in most instances greater than the viscosity of joint fluids obtained from pathological knee joints. Because of the small amount of fluid obtained, the amount of hyaluronic acid present in these normal joint fluids cannot at the present time be determined by chemical means. However, the high viscosity would indicate that these fluids contain either a large amount of hyaluronic acid per unit volume or that the polysaccharide is in a highly polymerized state or in a state of high aggregation. On standing at icebox temperature over a period of weeks in a sterile state, the viscosity of the pathological fluids does not change.[12] Thus, if a specific enzyme is present, it is in an inactive state when removed from the body.

Conclusions. 1. The viscosity of normal human knee joint fluid is greater than that of fluid found in most pathological conditions. 2. The decreased viscosity of joint fluids found in pathological states may be

11 Bauer, W., Ropes, M. W., and Waine, H., *Physiol. Rev.*, 1940, **20**, 272.

† We would like to visualize this space as the interfibrillar tissue space. In this sense it is extracellular.

12 Robertson, W. VanB., Ropes, M. W., and Bauer, W., *J. Biol. Chem.*, 1940, **133**, 261.

TABLE II.
Inactivating Effect of Pepsin.

	Virus conc.	1:20		1:200		1:500	
pH	Incubation time, hr	Active pepsin	Inactivated pepsin	Active pepsin	Inactivated pepsin	Active pepsin	NaCl-HCl controls*
2.0	½	9/10	10/10	3/10	6/10	9/10	
"	1	0/10	1/10	0/10	2/10	3/10	
"	2	0/10	0/10	0/10	0/10	0/10	
3.0	½					9/10	
"	1					7/10	
"	2					6/10	9/10

* For other comparative figures with NaCl-HCl-virus mixtures, see Table I.

the normal fasting gastric contents, virus is rapidly and completely inactivated. At somewhat lower levels (pH 2.0-3.0) pepsin has a slight inactivating effect. However, the actual effectiveness of this "barrier" in preventing the passage of virus into the intestine is certainly far from complete since the optimal conditions for inactivation are present in the stomach only at times. The ingestion of milk raises the pH of the contents and maintains it, at least in infants up to 19 months, at 3.2 or more for at least 2 hours.[11] With carbohydrate foods (Ewald test meals) on the other hand pH levels are frequently at the inactivating levels. Thus, Kahn and Stokes[12] found in 5 human subjects aged 4, 5, 6, 9 and 43 years old respectively with gastrostomies that after one hour, the pH was 1.32, 1.56, 1.57, 1.73 and 3.31. At the height of digestion of an ordinary meal containing meat, the acidity of the gastric contents is stated to lie in the range pH 1.3-2.5.[9] Mucus and saliva are said to raise the pH somewhat.[13] The rate of gastric evacuation is another factor of importance. After ingestion of most foods, evacuation begins in 1-7 minutes, and with fluids a considerable amount may leave the stomach almost at once. On the other hand, emptying requires 3 hours or more, being especially rapid with carbohydrates (95% at 3 hours), a little less so with proteins (80-85% at 3 hours) and quite slow with fats (30-50% in 3 hours).[9]

Taking the varying conditions of gastric acidity and motility into consideration it seems clear that when virus is swallowed, some of it is destroyed in the stomach and some enters the intestine intact, but the proportions cannot be well estimated. Conditions of high acidity, such for instance as occur after ingestion of carbohydrates and meats, favor inactivation while highly buffered foods, particularly milk, tend to prevent it. Virus contained in that part of the ingesta which leaves the stomach too soon to be highly acidified is apt to escape inactivation.

Since the pH of the intestine has a range of about 6.0-8.0[14] and since, as we have shown, trypsin has no inactivating effect, any virus reaching the intestine is likely to remain intact, and to have the opportunity of causing infection. Virus can also become concentrated in the large bowel as water is abstracted from the contents and thus become easily demonstrable in the stools. While, as we[15] have mentioned elsewhere, we believe that primary intestinal entry is an exceptional event, we suspect on the basis of the frequency of lesions in the celiac ganglia[15] that secondary intestinal entry following pharyngeal entry is not uncommon.

Summary. At the pH levels prevailing in

[11] Babbott, F. L., Jr., Johnston, J. A., Haskins, C. H., and Shohl, A. T., *Am. J. Dis. Child.*, 1923, **26**, 475.

[12] Kahn, G., and Stokes, J., Jr., *Am. J. Dis. Child.*, 1926, **32**, 667.

[13] Marriott, W. McK., and Davidson, L. T., *Am. J. Dis. Child.*, 1923, **26**, 542.

[14] Mann, F. C., and Bollman, J. L., *J. Am. Med. Assn.*, 1930, **95**, 1722.

[15] Faber, H. K., and Silverberg, R. J., *J. Exp. Med.*, 1946, **83**, 329.

stored in ampoules on dry ice, and appropriately diluted with saline solution at the time of use. Swiss mice, all, with a few exceptions, of the Webster strain from the Carworth Farms, were employed, using a standard intracerebral inoculum of 0.03 cc. Positive results were determined either by the appearance of paralysis or, in the case of mice found dead without observed symptoms, by histological examination. While differing from the current practice of judging results by the gross fatality rate, our method somewhat increases the accuracy and significance of the results by eliminating deaths from nonpoliomyelitic causes. In the tables, the numerator expresses the number of mice showing poliomyelitis and the denominator, the number of mice tested. pH determinations were made electrometrically, both before and after incubation of each preparation. Mixtures were adjusted with HCl within the range pH 1.0-3.0; for pH 4.0, an acetic acid-NaOH mixture was used. At the end of incubation at 37°C, each preparation was returned to approximate neutrality by the addition of NaOH. In the pepsin experiments, acid solutions of Lilly's pepsin were made up to contain 12% pepsin in the final mixture with virus and adjusted to the desired pH The proteolytic activity of each pepsin preparation was tested on coagulated egg albumin. At the end of each incubation period, enzyme activity was stopped by the addition of NaOH to approximate neutrality. For the controls, pepsin inactivated at 70°C for ½ hour was used in the same amounts as in the tests. (Results shown in Tables I and II).

TABLE I.

Inactivation of Lansing Virus at Varying pH Levels.

1:500 suspensions.

	Incubation time					
pH	5 min	¼ hr	½ hr	1 hr	2 hr	4 hr
1.0	0/7	0/7	0/7	0/7	0/7	0/6
1.5	3/6	0/6	0/6	0/6	0/6	0/6
2.0	6/7	4/7	5/7	4/7	3/6 (0/13)*	0/6
3.0	6/8	6/7	6/8	6/7	5/8 (14/19)*	8/8
4.0	7/8	7/8	4/8	5/8	5/8	6/8

* Another series of tests.

This group of experiments shows complete inactivation of virus in 5 minutes at pH 1.0. At pH 1.5, partial inactivation occurs at 5 minutes, and complete at 15 minutes, while at pH 2.0, partial inactivation begins at about 2 hours and is complete at 4 hours. At pH 3.0 and pH 4.0, no inactivation is found at 4 hours. In other tests not tabulated, inactivation was complete at pH 3.0 in 22 hours, but incomplete at pH 4.0 in the same period.

The data indicate a slightly greater degree of inactivation with pepsin than by acidity alone, but the difference between tests and controls is small. The optimum proteolytic effect of pepsin is stated to occur at pH 2.0-2.8.[10]

Effect of Trypsin. Supplementing the study of gastric digestion on virus, the effect of trypsin was investigated. For technical reasons, mice could not be used for this purpose and the experiments were performed on monkeys with a freshly isolated strain of poliomyelitis virus in its first passage. A suspension made up to contain 10% pancreatin (Parke-Davis) and 10% virus adjusted to pH 7.9 was incubated for 4½ hours at 37°C. The activity of the pancreatin was confirmed by visible digestion of egg albumin and by determination of pH and free acids in casein digest. At the end of incubation pancreatic activity was inhibited by lowering the pH to 4.2 by the addition of HCl. In 6 Rhesus monkeys, 0.5 cc of the virus-pancreatin mixture was instilled daily into each nostril for 5 consecutive days. Three monkeys developed typical paralytic poliomyelitis, and one, nonparalytic poliomyelitis as demonstrated by characteristic lesions in the cord. Two monkeys showed no evidence of infection. Of 6 controls given virus in saline suspension by the same procedure, none developed clinical signs of poliomyelitis and the olfactory bulbs of 2 showed no lesions. It would appear that exposure to trypsin not only fails to destroy the virus but may even increase its infectivity.

Comment. Our experiments indicate that at high levels of acidity such as prevail in

[10] Northrop, J. H., *J. Gen. Physiol.*, 1919-20, **2**, 113.

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the stomach when only gastric juice is present, and at the height of digestion of carbohydrate and mixed meals containing meat, poliomyelitis virus is rapidly inactivated. Pepsin contributes slightly to inactivation. Since the pH levels necessary for virus inactivation are present in the stomach only part of the time, and since part of the gastric contents are evacuated before such levels can be attained, a certain proportion of ingested virus has the opportunity to escape intact into the duodenum; here and in the rest of the intestine the pH is too high to inactivate it and trypsin has no inactivating effect. Virus entering the bowel therefore must be presumed to remain active, thus providing conditions suitable for secondary intestinal entry of infection, and also for concentration of virus in the contents of the large intestine.

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15348

Toxicity of Solutions of Cocaine Hydrochloride of Various Ages.

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Although cocaine hydrochloride is a very useful drug, it is also very toxic. The frequency of untoward reactions has been decreased by premedication with one of the barbiturate series of drugs but recent reports[1,2] indicate that fatalities still occur following the use of cocaine. Several clinicians[3-6] have found that no serious reactions occurred when they used an absolutely fresh solution of the drug and this has been our clinical experience.

Regnier, David and Joriot[7] have reported that solutions of cocaine hydrochloride that were 6 months to several years old were more toxic to roots of white lupine seedlings than were fresh solutions but we have been unable to find a report of any investigation on animals of the relation between the age and the toxicity of solutions of cocaine hydrochloride. Consequently the following experiments were done.

Method and Results. Using sterile precautions, 1% solutions of cocaine hydrochloride in sterile distilled water were made and placed in stoppered bottles of clear glass. The solutions were kept at room temperature (75° to 95°F) exposed to daylight but not to the direct rays of the sun.

Healthy adult rabbits of both sexes were used; their weights varied from 1.5 to 4.0 kg. It was determined that the intravenous injection in 10 seconds of 10 mg of cocaine hydrochloride per kilo of body weight, a 1% solution being used, was always followed by a generalized convulsion and fairly frequently by death. This dosage and technic of injection were followed throughout this experiment.

The fresh solution of cocaine hydrochloride was used during the first hour after it was prepared. The toxicity of this solution was compared with that of solutions one week, one month and 3½ months old. It seemed wise to compare the lethal effects, as the accurate

1 Derbes, V. J., and Englehardt, H. T., *J. Lab. and Clin. Med.*, 1944, **29**, 478.

2 Shumaker, H. B., Jr., *Sur.*, 1941, **10**, 134.

3 Gardner, J. A., *J. Urol.*, 1923, **10**, 509.

4 Braasch, W. F., quoted by Hirschfelder, A. D., and Bieter, R. N., *Physiol. Rev.*, 1932, **12**, 190.

5 Emmett, J. L., *Local Anesthesia of the Urethra and Bladder*; in Lundy, J. S., *Clinical Anesthesia*, Philadelphia, W. B. Saunders Co., 1942, 195.

6 Moersch, H. J., personal communication.

7 Regnier, Jean, David, Robert, and Joriot, Robert. *Comp. rend Soc. de biol.*, 1937, **125**, 1012.

TABLE I.
Adsorption of Zephiran on Various Surfaces.

Solution	Surface tension
Water	72.8 dynes
50 ppm Zephiran	66.5
80 " "	62.5
100 " "	59.2
Same + 1.3% filter paper	62.2
Same + 1% bakers yeast	63.4
1000 ppm Zephiran	37
Same + 5% bentonite	73

solution increased noticeably in volume, suggesting thick adsorption layers. Such layers may retard the diffusion of dissolved molecules to the center of a clump of bacteria.

However, another fact must also be included into any explanation, namely the observation by Miller, Abrams, Huber, and Klein[2] that hands dipped in 1% Zephiran solution remain apparently sterile for about 2 hours through an imperceptible film, while the bacteria of the skin, under this film, are still alive when the film is disrupted several hours later.

This observation can be explained by assuming that the adsorbed Zephiran molecules or ions are oriented, the inactive, harmless organic end directed towards the skin and the germicidal NOH group towards the outside. The organophilic skin attracts the long organic chains, and thus, the adsorption film has one harmless side and one toxic side.

To test this explanation, experiments were made with 2 different surfaces, namely fat which is organophilic, and 25% gelatin which is hydrophilic. About 1 to 2 g butterfat of high melting point were pipetted into test-tubes, and after solidification, 0.1 ml of a Staphylococcus culture was put upon the fat surface. When the culture was perfectly dry (in a vacuum), 2 to 5 ml of a 1% Zephiran solution were poured on the dry bacteria. After various exposure times, the disinfectant was poured off, the entire tube was washed once with 50 ppm Duponol (a good antidote to Zephiran), then twice with 10 ppm Duponol, and finally, broth containing 5 ppm Duponol was poured into the tube. The fat surface was then scraped with a bent platinum wire to remove as many bacteria as possible, the tube was shaken violently, and 1 ml and 0.01 ml of the broth was plated. The counts thus obtained are extremely inaccurate because bacteria may not all be removed, or may become imbedded in the fat. The chance of contamination by the repeated washing is increased. The recovery of bacteria dried on fat and treated with water gave between 200 and 20,000 colonies. If only very few colonies are found, and not consistently, it suggests a chance contamination while larger numbers are good proof of survival.

[2] Miller, B. F., Abrams, B., Huber, D. A., and Klein, M., PROC. SOC. EXP. BIOL. AND MED., 1943, 54, 174.

TABLE II.
Survivors of *Staphylococcus aureus* Exposed to Zephiran Solution in Presence of Various Surfaces.

Surface	Butterfat		
Disinfectant	1% Zephiran		
Bacteria	Dried I	Dried II	Moist II
0.5 hr	12,100	—	—
1	4,000	170	—
1.5	50,000	—	—
4	—	190	0
20	—	30	0

Surface	25% gelatin				
Disinfectant	1% Zephiran	0.1% Zephiran		0.01% Zephiran	
Bacteria	dry	dry	moist	dry	moist
0.5 hr	0	0	0	1:590	9,700
1	0	0	—	0	—
1.5	0	0	0	900	0
4	0	0	—	0	—

TABLE I.
Toxicity of Solutions of Cocaine Hydrochloride.*

Age of solution	Rabbits	Deaths	Mortality rate %
Fresh	76	15	19.7
1 wk	87	25	28.7
1 mo	36	9	25.0
3½ "	10	1	10.0

* The drug was injected intravenously in 10 seconds in the dosage of 10 mg per kilo of body weight, a 1% solution being used.

grading of convulsions seemed impossible (Table I). It will be seen that there was not a demonstrable difference in the toxicity of the solutions of various ages. There was no evidence that the sex or the weight of the rabbits had any influence on the mortality rate. Neither was there any evidence that the fresh solution of cocaine hydrochloride became more toxic during the hour-long period that it was being used.

These results do not support the clinical impression that fresh solutions of cocaine hydrochloride are less toxic than older ones. However, it has been shown that sensitivity to cocaine hydrochloride varies among members of the animal kingdom, the monkey being much more sensitive than the rabbit.[8] It seems probable that the human being is even more sensitive. If this is true, then it seems possible that small differences in toxicity which could not be detected in the rabbit might be of real significance in human beings. Therefore, even though the older solutions of cocaine hydrochloride did not cause a higher death rate in rabbits, it should not be concluded that old solutions of cocaine are entirely safe for human beings. In view of the clinical reports cited in the earlier part of this paper, it would seem wise to use fresh solutions of cocaine exclusively in clinical work.

Summary. The toxicity of solutions of cocaine hydrochloride of various ages (less than one hour, one week, one month and 3½ months) was studied in rabbits, the drug being given intravenously in the dosage of 10 mg per kilo of body weight. The mortality rates following injections of solutions of cocaine of various ages showed no significant differences.

[8] Tatum, A. L., and Collins, A. H., *Arch. Int. Med.*, 1926, **38**, 405.

15349

Protection of Dry Bacteria by Fat Against Cationic Detergents.

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Klarmann and Wright[1] have shown that quaternary ammonium compounds cause an agglutination of bacteria and precipitate them against the walls of the test tube. This causes a very coarse and irregular distribution and explains the irregular results obtained by every investigator trying to evaluate the germicidal efficiency of these compounds.

It is not clear, however, why the bacteria in these clumps die more slowly than those which remain singly in suspension. In order to prove whether these detergents are strongly adsorbed on the surfaces of bacteria, 1 g of baker's yeast was suspended in 100 ml of a 100 ppm solution of Zephiran (dimethylbenzyl alkyl ammonium chloride, also known as Röccal), the yeast removed by centrifugation, and the surface tension of the clear solution measured by the drop number method (Table I.) Nearly 20 ppm of the Zephiran had been adsorbed on the cells. This amount indicates a multimolecular adsorption layer. Bentonite particles in 1000 ppm Zephiran

[1] Klarmann, E. G., and Wright, E. S., *Soap and San. Chem.*, 1946, **22**, 125.

15350 P

Alloxan-Induced Azotemia in the Rat.*

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The production of renal injury by alloxan is well known and was pointed out by Dunn and McLetchie[1] in their original article on the production of alloxan diabetes in the rat. In the dog, the islet tissue is more susceptible to injury than is the kidney and thus smaller doses of alloxan are nearly selectively diabetogenic in this species. With larger doses, as Goldner and Gomori[2] have shown, sufficient renal damage is produced to cause a diabetic uremic syndrome. These experiments were undertaken to determine whether this differential sensitivity was present in the rat, and whether a dose level of alloxan could be found that would produce hyperglycemia without azotemia.

All animals used were male rats of the Long-Evans strain between 45 and 50 days of age at the time of the initial injection, and were allowed free access to the stock diet at all times. Alloxan monohydrate in aqueous solution was used and was injected intraperitoneally on a body weight basis. Animals were sacrificed 72 hours after the initial injection except in those cases where the effects of persistent diabetes were studied, in which case they were sacrificed approximately one month after the initial injection. Blood was obtained from the inferior vena cava and following deproteinization, glucose was determined by the Somogyi[3] micro-method and non-protein nitrogen was determined by the micro-Kjeldahl procedure.

In Table I are presented the data on the blood glucose and blood NPN obtained 72 hours after the initial injection of various amounts of alloxan. It will be seen that with a dosage of 200 mg per kg there was a slight rise in the mean glycemia and the azotemia although all animals did not respond. The degree of elevation of the NPN was somewhat greater than that of the blood glucose, one animal showing a blood NPN of 153 mg %, as contrasted with 34 mg %, the maximum of the control group. At a dose level of 250 mg per kg the mean blood glucose was approximately doubled, being 219 mg %; but the mean NPN was increased approximately four-fold being 115 mg %. With larger doses whether given as a single injection or as repeated smaller injections, both the hyperglycemia and azotemia were more marked. In general the severity of the histopathological changes in the kidney paralleled the degree of nitrogen retention.

That this effect on the nitrogenous constituents of the blood was due to a primary renal injury and was not due to functional renal failure secondary to the severe diabetic state was shown by the fact that the simultaneous administration of insulin prevented the extreme hyperglycemia but did not alter the azotemia.

In another series of animals which received 400 mg of alloxan per kg it was determined that 3 major components of the NPN: namely, urea N, uric acid N, and creatinine N were all elevated. The mean control urea N level was 22 mg % as compared with a mean experimental level of 165 mg %. For uric acid and creatinine the control values were 3.2 mg % and 1.7 mg % respectively as compared with 12.5 mg % and 6.7 mg % for the alloxan-treated group.

* Aided by grants from the Research Board of the University of California, and the James Foundation Grant of the Medical School.

1 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

2 Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

3 Somogyi, M., *J. Biol. Chem.*, 1937, **117**, 771.

TABLE III.
Survivors of *Staphylococcus aureus* Exposed to Disinfectants in Presence of Various Surfaces.

Surface	Vaspar		Fat		Glass	
Bacteria	dried	moist	dried	moist	dried	moist
	Disinfectant: 8% formaldehyde					
0.5 hr	0	0	261	—	0	4
1	0	0	14	0	0	1
2	900	1	0	—	—	0
	Disinfectant: 1% ceepryn					
0.5	4,400	4	—	0	0	1
1	3,500	0	24,700	42	0	0
2	—	23	—	—	—	—
3.5	24	—	0	—	0	0
7	7	—	22	—	0	—
22	0	—	0	—	—	—

The procedure with gelatin was similar, except that finally, the gelatin was melted in the broth at 40°, and plated directly. The result is shown in Table II. Bacteria dried on fat survived the powerful disinfectant for 20 hours, while they died within half an hour when not dried. By the standard technic, 50 ppm kill *Staphylococcus aureus* in 10 minutes. Bacteria dried on gelatin did not survive half an hour. Evidently the 2 surfaces affected the bacteria differently.

Table III gives the results of a parallel experiment with Ceepryn (cetyl pyridinium chloride), and with formaldehyde. Fat exerted a definite protection against formaldehyde, but not as pronounced as against Ceepryn. Vaspar (vaseline-paraffine mixture) as a pure hydrocarbon is even more organophilic than fat. The glass surface offered no protection. Bacteria dried on sand were also killed as easily as without sand.

Mudd and Mudd[3] have shown that bacteria are drawn to the interface between oil and water and usually remain there. Only the acid-fast bacteria are drawn into the oil phase. However, Jensen[4] states: "If sodium oleate in 0.05% solution is added to the water, every species and kind of microbes pass into the oil." A Zephiran solution will probably exert an influence similar to that of sodium oleate. Although the fat was solid, it is possible that a very thin film of fat covered some of the dry bacteria. Or perhaps, the surface of bacteria is so changed by drying that it becomes organophilic and becomes saturated with fat if that is present. Such cells would be protected against quaternary ammonium compounds because they would adsorb the harmless end of the molecule.

Summary. The adsorption of quaternary ammonium compounds on cell surfaces has been proved experimentally.

The formation, on hands, of a film of cationic detergent of which only the outside is germicidal, has been explained as due to an oriented adsorption of the detergents on an organophilic surface, such as fat or paraffin.

[3] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, **40**, 659.

[4] Jensen, L. B., *Microbiology of Meats*, Garrard Press, 1945, 286.

15351

Failure of Methionine to Reduce Nitrogen Loss in Postoperative Herniorrhaphy Patients on Restricted Diet.*

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Evidence that the addition of methionine to a standard diet decreased the urinary nitrogen output of dogs was reported by Miller.[1] It seemed of interest to see whether a similar effect could be obtained in patients, particularly surgical patients during the period of reduced food intake which often follows after operation.

For reasons of availability and relative uniformity 14 male patients were selected who were undergoing unilateral herniorrhaphy. They were divided into 3 groups as follows:

1. Four controls were studied for 10 days beginning on the first postoperative day. They received no added methionine.

2. Five patients were studied for 10 days beginning on the first postoperative day. They received 6.0 g of methionine daily for the first 5 days of the study.

3. Another 5 patients were studied for 10 days beginning on the first postoperative day. They received 6.0 g of methionine daily for the second 5 days of the study.

All patients were fed daily a diet containing approximately 1,000 calories and consisting of 40.0 g of protein, 40.0 g of fat, and 130.0 g of carbohydrate. The patients were kept in bed throughout the study.

Urine was collected in 24-hour periods and the volume measured. Aliquots of the first 5 days were pooled, and aliquots of the second 5 days were pooled. Analyses were run on a portion of each pooled specimen for total nitrogen by a semi-micro Kjeldahl method. Total urinary nitrogen excretion was calculated from this. Fecal nitrogen output was not determined in this group of patients but averaged 1.0 g in 18 other patients studied.[2] In this study we used an average figure of 1.0 g daily.

Results. Table I shows average intake and average excretion of nitrogen in the individual patients in the 3 groups for the 2 5-day periods.

Plasma protein concentrations were not significantly altered during the 10-day period.

Discussion. As no significant difference was found in the controls between the mean difference in intake and output of nitrogen for the first 5 days and the mean difference in intake and output of nitrogen for the second 5 days when no methionine was given, any significant differences found in those patients receiving methionine could properly be attributed to the effect of methionine. However, although small differences were found in the average output between the "methionine-fed" period and the "no methionine" period, when analyzed statistically the differences were found not to be significant (Table I). The amount of methionine fed these patients (approximately 0.1 g per kilo of body weight) was in the same range as the dogs studied by Miller.[1] The patients were on a low total caloric diet and a low, but not starvation, intake of nitrogen. It was found by Miller, whose dogs presumably received sufficient calories for maintenance, that the effect of methionine was greater in his animals before their protein stores had become depleted. These patients prior to operation were on a normal diet, and it must

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

[1] Miller, L. L., *J. Biol. Chem.*, 1944, **152**, 603.

[2] Riegel, Cecilia, Koop, C. E., Drew, J., Stevens, L. W., and Rhoads, J. E., to be published.

TABLE I.
Blood Glucose and Non-protein Nitrogen Level in the Rat 72 Hours After the Initial Alloxan Administration.

Alloxan dose per kg	Blood glucose in mg %		Blood NPN in mg %	
	Mean	Range	Mean	Range
Uninj. control	129 (13)*	113-146	27 (8)	23-34
150 mg	118 (13)	85-155	32 (6)	25-39
200 "	151 (11)	108-333	66 (6)	26-153
250 "	219 (10)	123-388	115 (4)	27-215
300 "	381 (6)	156-648	201 (4)	168-235
150 " 2 consecutive days. Total dose 300 mg	354 (13)	129-668	115 (6)	61-233
200 mg 2 consecutive days. Total 400 mg	736 (8)	149†-1030	140 (8)	41-238
150 mg 3 consecutive days. Total 450 mg	549 (8)	189-955	185 (8)	95-347
200 mg 2 consecutive days plus 2 units protamine insulin daily	140 (8)	41-238	177 (5)	83-245

* The figure in parentheses indicates the number of observations in the group.
† Only one value below 600 mg %.

TABLE II.
Blood Glucose and Non-protein Nitrogen Level in Rats with Alloxan Induced Diabetes of One Month's Duration.

	Blood glucose in mg %		Blood NPN in mg %	
	Mean	Range	Mean	Range
Uninjected controls (7)*	122	76-143	27	22-30
Persistent diabetics (8)	431	381-510	41	24-70
Recovered diabetics (5)	132	111-153	30	27-31

* The figure in parentheses indicates the number of observations in the group.

In Table II are presented the data obtained from animals 31-34 days following the administration of 400 mg of alloxan per kg. It will be seen that even in those animals in which there was a persistent diabetes, there was only slight residual azotemia. The mean blood NPN in this group was 41 mg % as compared with 27 mg % for the controls or as compared with 140 mg % for animals 72 hours after the injection of the same amount of alloxan. This would seem to indicate a high degree of functional recovery of the kidney. In those animals in which diabetes was not persistent there was a complete functional recovery of the kidney insofar as the NPN was completely normal.

Summary. In rats of the Long-Evans strain there is no difference between the diabetogenic and nephrotoxic dose of alloxan as judged by hyperglycemia and azotemia. Even when diabetes persists there is nearly complete functional recovery of the kidney.

the same time in a report by Weiss[2] from this laboratory. Recently Dienes[3] has reviewed the morphology and nature of the entire pleuropneumonia group of organisms including rat strains such as we have used.

During the last 2 years we have tried out a rather long list of drugs, etc., in experimental chemotherapy in this field with no positive results until we recently tried streptomycin with what seems to be considerable success. The purpose of this report is to present these results showing effectiveness of streptomycin in this type of arthritis of rats.

In a routine screening test of various drugs, 0.05 cc of the same pleuropneumonia culture as used previously, and grown as described,[1] was injected intravenously into a group of white rats each of about 100 g weight. Four of these rats were treated hypodermically with streptomycin, while 6 were left as controls. Streptomycin therapy was started about an hour following infection, and 3 doses were given the first, second, and third days, or a total of 9 doses. Each dose comprised 1000 units. In this test, the 4 treated rats remained entirely free of symptoms. Five of the 6 controls developed pleuropneumonia infection, and one remained free of perceptible symptoms. Two of the 5 controls which developed infection had an overwhelming disease and were dead in 5 days, before very definite gross arthritis could appear. The other 3 controls developed disabling arthritis. All surviving animals were observed for 3 weeks. This kind of chemotherapeutic showing had not been seen previously in any pleuropneumonia-infected rats except those treated earlier with myochrysine, and the drug in this case was very toxic. By contrast the 4 streptomycin-treated rats in the present experiment appeared bright and alert, and entirely normal for 3 weeks after infection.

This initial experiment was repeated using 20 rats injected with pleuropneumonia culture as before. Ten of these were treated hypodermically with streptomycin and 10 were left as controls. Three doses of streptomycin were given on the first, second, third, and fourth days, or a total of 12 doses. Each dose comprised 3000 units. In this test 8 of the 10 treated rats remained entirely free of symptoms, while the remaining 2 showed a questionable trace of swelling in one toe of each animal for 2 days only. All 10 treated rats were bright and alert throughout the test and exhibited no evidence of drug toxicity. All 10 of the control rats, however, showed early and pronounced symptoms of pleuropneumonia infection. Three of these died in 5 days of overwhelming disease before visible arthritis usually appears. The other 7 developed disabling polyarthritis and died before the 3-weeks observation period was over. It is believed that the good showing of streptomycin in this experiment has not been equalled previously with myochrysine in our tests.

A third experiment is in progress using 33 rats injected with pleuropneumonia culture as before. Sixteen of these were treated with streptomycin and 17 were left as controls. None of the 16 treated rats show any symptoms, while all of the controls show evidence of infection (rapid respiration, loss of weight, and disinclination to move about), and beginning arthritis is apparent in these.

Further details concerning the effectiveness of streptomycin in rat arthritis are under study.

Our thanks are due Miss Dorothy McKay for cooperation and interest in the experimental chemotherapy set forth in this report.

Summary. Streptomycin appears chemotherapeutically effective against pleuropneumonia infections and resultant polyarthritis in rats, and better in this respect than certain gold salts. The streptomycin which we used contained 250 units per mg, and was supplied by Dr. J. A. Leighty.

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[2] Weiss, L. J., *J. Bact.*, 1944, 47, 523.

[3] Dienes, L., *J. Bact.*, 1945, 50, 441.

TABLE I.
Effect of Administration of Methionine on Urinary Nitrogen Excretion.

	First 5 days			Second 5 days		
	Intake g	Urine, N. g	Diff. g	Intake g	Urine, N. g	Diff. g
			No methionine			
1	32.8	62.2	—29.4	33.6	50.9	—17.3
2	35.3	61.8	—26.5	32.0	47.1	—15.1
3	35.3	49.3	—14.0	32.4	44.7	—12.3
4	33.5	57.6	—24.1	33.1	57.1	—24.0
	Mean diff.		—23.5	Mean diff.		—17.1
			Methionine first 5 days—6.0 g daily			
5	35.9	36.3	—0.4	32.8	31.1	+1.7
6	35.0	69.1	—34.1	32.8	72.2	—39.4
7	35.1	68.1	—33.0	32.1	54.7	—22.6
8	32.2	63.0	—30.8	33.9	79.2	—45.3
9	35.7	71.6	—35.9	31.2	60.7	—29.5
	Mean diff.		—26.8	Mean diff.		—27.7
			Methionine second 5 days—6.0 g daily			
10	32.4	63.4	—31.0	36.0	51.7	—15.7
11	33.0	38.8	—5.8	35.5	44.1	—8.6
12	31.9	57.9	—26.0	35.4	62.7	—27.3
13	32.9	46.1	—13.2	33.9	44.1	—10.2
14	30.1	39.5	—9.4	32.9	46.6	—13.7
	Mean diff.		—17.1	Mean diff.		—15.1

Controls—First 5 days — mean difference 23.5 g
Second 5 " — " " 17.1 g

Diff. between means = 6.4 g S.E. diff. = 4.1

Methionine Exper.—Period with methionine M.D. 21.0 g
" without " M.D. 22.4 g

Diff. between means = 1.4 g S.E. diff. = 5.9

be presumed that their protein stores were not depleted.

Conclusion. Under the conditions of this experiment the administration of 6.0 g of methionine daily to men receiving a low caloric diet failed to show a significant protein-sparing action as indicated by the effect on urinary nitrogen excretion.

15352 P

Effectiveness of Streptomycin in Arthritis of Rats.

H. M. POWELL, W. A. JAMIESON, AND R. M. RICE.
From the Lilly Research Laboratories, Indianapolis, Ind.

Two years ago we reported that penicillin was ineffective in the chemotherapy of arthritic rats infected with pleuropneumonia-like organisms, while myochrysine was effective in this respect, but at the same time quite toxic and hazardous to use in these animals.[1] Electron morphology of the micro-organisms we used was dealt with at about

[1] Powell, H. M., and Rice, R. M., *J. Lab. and Clin. Med.*, 1944, **29**, 372.

the same time in a report by Weiss[2] from this laboratory. Recently Dienes[3] has reviewed the morphology and nature of the entire pleuropneumonia group of organisms including rat strains such as we have used.

During the last 2 years we have tried out a rather long list of drugs, etc., in experimental chemotherapy in this field with no positive results until we recently tried streptomycin with what seems to be considerable success. The purpose of this report is to present these results showing effectiveness of streptomycin in this type of arthritis of rats.

In a routine screening test of various drugs, 0.05 cc of the same pleuropneumonia culture as used previously, and grown as described,[1] was injected intravenously into a group of white rats each of about 100 g weight. Four of these rats were treated hypodermically with streptomycin, while 6 were left as controls. Streptomycin therapy was started about an hour following infection, and 3 doses were given the first, second, and third days, or a total of 9 doses. Each dose comprised 1000 units. In this test, the 4 treated rats remained entirely free of symptoms. Five of the 6 controls developed pleuropneumonia infection, and one remained free of perceptible symptoms. Two of the 5 controls which developed infection had an overwhelming disease and were dead in 5 days, before very definite gross arthritis could appear. The other 3 controls developed disabling arthritis. All surviving animals were observed for 3 weeks. This kind of chemotherapeutic showing had not been seen previously in any pleuropneumonia-infected rats except those treated earlier with myochrysine, and the drug in this case was very toxic. By contrast the 4 streptomycin-treated rats in the present experiment appeared bright and alert, and entirely normal for 3 weeks after infection.

This initial experiment was repeated using 20 rats injected with pleuropneumonia culture as before. Ten of these were treated hypodermically with streptomycin and 10 were left as controls. Three doses of streptomycin were given on the first, second, third, and fourth days, or a total of 12 doses. Each dose comprised 3000 units. In this test 8 of the 10 treated rats remained entirely free of symptoms, while the remaining 2 showed a questionable trace of swelling in one toe of each animal for 2 days only. All 10 treated rats were bright and alert throughout the test and exhibited no evidence of drug toxicity. All 10 of the control rats, however, showed early and pronounced symptoms of pleuropneumonia infection. Three of these died in 5 days of overwhelming disease before visible arthritis usually appears. The other 7 developed disabling polyarthritis and died before the 3-weeks observation period was over. It is believed that the good showing of streptomycin in this experiment has not been equalled previously with myochrysine in our tests.

A third experiment is in progress using 33 rats injected with pleuropneumonia culture as before. Sixteen of these were treated with streptomycin and 17 were left as controls. None of the 16 treated rats show any symptoms, while all of the controls show evidence of infection (rapid respiration, loss of weight, and disinclination to move about), and beginning arthritis is apparent in these.

Further details concerning the effectiveness of streptomycin in rat arthritis are under study.

Our thanks are due Miss Dorothy McKay for cooperation and interest in the experimental chemotherapy set forth in this report.

Summary. Streptomycin appears chemotherapeutically effective against pleuropneumonia infections and resultant polyarthritis in rats, and better in this respect than certain gold salts. The streptomycin which we used contained 250 units per mg, and was supplied by Dr. J. A. Leighty.

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2 Weiss, L. J., *J. Bact.*, 1944, 47, 523.

3 Dienes, L., *J. Bact.*, 1945, 50, 441.

15353

Presence of a Growth Inhibiting Substance in Raw Soybeans.

A. A. KLOSE, BARBARA HILL, AND H. L. FEVOLD.

From the Western Regional Research Laboratory, Albany, California, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

The low nutritional value of raw soybean proteins and the marked improvement on heating have been recognized for some time;[1,2,3] however, the fundamental mechanism by which this change takes place has not been satisfactorily explained. The experimentally demonstrated effect of heating is to make the methionine of the protein nutritionally available. Johnson, Parsons, and Steenbock[4] have shown that this effect is not brought about by increased absorption of the sulfur-containing amino acids but that heating apparently increases the utilization of these amino acids after absorption. Ham and Sandstedt[5] and Bowman[6] have demonstrated the presence of a trypsin inhibitor in the pH 4.2-soluble fraction of unheated soybean flakes, and Ham and Sandstedt suggested its possible significance in relation to the poor biological value of the raw soybean protein. They were not able at that time to demonstrate any growth-inhibiting effect of the fraction containing the trypsin inhibitor.

Recently, while this paper was in the process of review, a report by Ham, Sandstedt, and Mussehl[7] appeared which extended their observations and established a growth-retarding effect on chicks of the pH 4.2-soluble, acetone-insoluble fraction of soybeans. We had obtained similar results with rats, and these results are presented in this report.

In connection with work in progress at the Northern Regional Research Laboratory, dealing with the effects of processing on the food value of soybean proteins, we have been investigating the effects of heating on the growth-supporting properties of the separated acid-soluble (pH 4.2) and acid-insoluble proteins. In this investigation it was found that the crude pH 4.2-insoluble protein still responded to heat treatment by increased biological value. However, when the unheated acid-soluble fraction was added back to the heated acid-insoluble protein, the growth-promoting property was again reduced to that of unheated soybean proteins. It is obvious, therefore, that a growth-inhibiting substance is present in unheated soybean proteins which explains partially at least the nutritional inadequacy of raw soybean proteins.

Litters of albino rats (Sprague Dawley strain) were maintained on a stock diet of Purina Dog Chow Checkers supplemented with cod-liver oil and liver until they were approximately 40 days of age and 90 g per rat in weight. Each litter was then divided equally as far as possible among the experimental groups of 10 rats each and fed the corresponding diets and water *ad libitum*.

The soybean protein fractions were prepared in the following manner: ground hexane-extracted soybean flakes (No. 113) were stirred in a large volume (10 cc per g of flakes) of distilled water, and the mixture was adjusted to a pH of 6.7 with dilute sodium hydroxide. The resulting extract was pressed out in a small cider press and then

1 Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, **32**, 91.

2 Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219; **12**, 275.

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4 Johnson, L. M., Parsons, H. T., and Steenbock, H., *J. Nutrition*, 1939, **18**, 423.

5 Ham, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

6 Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 139.

7 Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

TABLE I.

Effect of Various Soybean Protein Supplements on Average Gain in Weight of Young Rats. (5 Males + 5 Females per Group).

Diet No.	Protein supplement equivalent to 14.6% crude protein	Experiment No. I 35-day test: Avg gain g/rat/day	I 35-day test: Feed-consumption, g/rat/day	I 35-day test: Gain per g protein eaten, g	II 28-day test: Avg gain g/rat/day	II 28-day test: Feed-consumption, g/rat/day	II 28-day test: Gain per g protein eaten, g	III 18-day test: Avg gain g/rat/day	III 18-day test: Feed-consumption, g/rat/day	III 18-day test: Gain per g protein eaten, g
1	Hexane-extracted soybean flakes No. 113	1.06	10.8	0.65	1.19	11.7	0.67			
2	No. 113 steamed	2.20	12.0	1.21	2.80	12.7	1.45			
3	pH 4.2 insoluble soybean protein No. 113A	0.82	9.8	0.55	1.27	11.4	0.73			
4	No. 113A steamed	2.05	12.0	1.13	1.82	11.8	1.02	2.41	12.3	1.29
5	No. 113A " 90% pH 4.2 soluble soybean protein— No. 113B 10%	1.12*	10.8	0.68	0.84	10.7	0.52	1.16	10.6	0.72
6	No. 113A steamed 95 No. 113B 5							1.70	11.7	0.96
7	No. 113A " 90 No. 113B " 10	2.06*	11.3	1.20	2.24	11.5	1.28			
8	No. 113A " 90 No. 113A 10							2.71	12.5	1.42
9	Casein, commercial†	2.93	12.9	1.49	3.22	13.2	1.60	3.57	11.5	2.04
10	Casein + No. 113B (1.5% crude protein)†							2.56	10.4	1.45
11	Casein + No. 113B steamed (1.5% crude protein)†							4.19	11.6	2.12

* Diet 7 was fed for 5 days to the group of rats which received Diet 5 for the first 30 days of the test.

† The data for 9, 10, and 11 (Exp. 3) are comparable, but not comparable with the rest of the data. Younger rats were used in these 3 groups.

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In connection with work in progress at the Northern Regional Research Laboratory, dealing with the effects of processing on the food value of soybean proteins, we have been investigating the effects of heating on the growth-supporting properties of the separated acid-soluble (pH 4.2) and acid-insoluble proteins. In this investigation it was found that the crude pH 4.2-insoluble protein still responded to heat treatment by increased biological value. However, when the unheated acid-soluble fraction was added back to the heated acid-insoluble protein, the growth-promoting property was again reduced to that of unheated soybean proteins. It is obvious, therefore, that a growth-inhibiting substance is present in unheated soybean proteins which explains partially at least the nutritional inadequacy of raw soybean proteins.

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15354

Urinary Output and Phosphorus Excretion in Human Subjects During Prolonged Exposures at Low Simulated Altitudes.*

SAVINO A. D'ANGELO. (Introduced by Harry A. Charipper).

From the Aero Medical Laboratory,† Wright Field, Dayton, Ohio, and the Department of Biology, Washington Square College of Arts and Sciences, New York University.‡

Among the many described effects of anoxia on the organism, those on the urinary secretion and the mineral metabolism are especially difficult of interpretation. Van Liere[1] has reviewed the evidence bearing on the problem and has indicated its controversial status. In neither the lower mammal nor in man are the results in good agreement. The anesthetized dog generally displays oliguria while breathing at reduced oxygen tensions equivalent to altitudes of 14,000 ft. and above, yet considerable variation and polyuria may occur.[2,3,4] In the unanesthetized animal at 18,000-20,000 ft. simulated altitudes polyuria appears to be the typical response.[5,6] Reports on the urinary output of the anoxic human being are also discordant. McFarland and Edwards[7] described increased urine elimination in certain of the crew, but not the passengers, at the beginning of prolonged trans-Pacific flights at altitudes of 8,000 to 12,000 ft. The diuresis was marked in the chronic anoxia experiments of Armstrong and Heim.[8] Urine output was increased from 100 to 300% of normal in subjects maintained for 4 or 7 hours daily at a 12,000 ft. simulated altitude. Bryan and Ricketts,[9] on the other hand, obtained essentially negative results at comparable as well as higher altitudes (11,500-18,000 ft.)

The scanty reports regarding the effect of altitude on the mineral metabolism are similarly conflicting. Acute anoxia in the dog produces a pronounced increase in the renal excretion of sodium, potassium, chloride, as well as lesser increases in the excretion of nitrogen and phosphorus.[5,6] These changes do not occur in the human subject. The species difference is particularly marked as regards phosphorus metabolism. Sundstroem,[10] Lewis *et al.*[6] and Bryan and Ricketts[9] have all demonstrated that phosphorus excretion in the human being is decreased rather than increased in anoxia. The physiological significance of this effect is still unclear. The present study represents one phase of a research project investigating the physiological responses of the human subject during prolonged exposures at moderately low simulated altitudes. The effects on the carbohydrate

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3 Toth, L. A., *Am. J. Phy.*, 1937, **119**, 127.

4 Malmejac, J., *J. Av. Med.*, 1944, **15**, 167.

5 Langley, L. L., and Clarke, R. W., *Yale J. Biol. and Med.*, 1942, **14**, 529.

6 Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.*, 1942, **21**, 33.

7 McFarland, R. A., and Edwards, H. T., *J. Av. Med.*, 1937, **8**, 156.

8 Armstrong, H. G., *Principles and Practices of Aviation Medicine*, Baltimore, Williams and Wilkins Co., 1939, 284.

9 Bryan, H. A., and Ricketts, H. T., *J. Clin. Endocrinol.*, 1944, **4**, 450.

10 Sundstroem, E. S., *Univ. California Publ. Physiol.*, 1919, **5**, 121.

centrifuged in a Sharples centrifuge to remove suspended insoluble material. A second extraction of the press cake was made in a similar fashion. The combined extracts were adjusted to pH 4.2 with dilute (1*N*) hydrochloric acid. In Exp. I the pH 4.2-insoluble material (No. 113A) was centrifuged relatively free of filtrate and then dried *in vacuo* from the frozen state. In Exp. II the pH 4.2-insoluble material was redissolved by addition of alkali and reprecipitated at pH 4.2 4 times, after which it was also dried by lyophilization. Sufficient ammonium sulfate was added to the combined supernatant filtrates from the centrifugation to bring the concentration to 2.7 *M*. The resulting precipitate (No. 113B) was separated by centrifugation, dialyzed in Visking casings against distilled water at 1°C until free of ammonium sulfate, and then dried *in vacuo* from the frozen state. An optimum heat treatment of the soybean protein was accomplished by steaming in an autoclave for 30 minutes at atmospheric pressure.

All diets were composed of 50% basal mixture, a percentage of protein supplement equivalent to 14.6% crude protein, and enough of a 50-50 mixture of starch and sucrose to make 100%. Crude protein percentage was calculated by using 6.38 × % *N* for casein and 5.71 × % *N* for the soybean proteins. The basal mixture, making up 50% of each diet, contained (as percent of the complete diet) cottonseed (Wesson) oil 5, U. S. P. cod-liver oil 2,34% tocopherol vegetable oil concentrate 0.05, salt mixture (McCollum's No. 185 + trace elements) 4, corn starch 18.5, sucrose 18.5, brewers' yeast 2, and (as mg per 100 g of the complete diet) choline chloride 50, thiamin chloride 0.2, riboflavin 0.5, pyridoxin 0.2, calcium pantothenate 2.5, and nicotinic acid 1.0.

Table I presents the results obtained on feeding the acid-soluble (pH 4.2) and acid-insoluble proteins in various combinations. The marked growth-retarding effect of the unheated, pH 4.2-soluble, 2.7 *M* ammonium sulfate-insoluble fraction when allowed to replace 10% of the heated pH 4.2-insoluble fraction and the dissipation of the effect on heating, is at once apparent. It is also obvious that the growth inhibitor is concentrated in the 10% of pH 4.2-soluble protein, since the effect of the inhibitor contained in 10% of soluble protein, when introduced in the diet, is greater than that of the inhibitor contained in 10 times the amount of pH 4.2-insoluble protein (compare 3, 4, and 5, Exp. II); also, the inclusion of 10% of unheated pH 4.2-insoluble protein results in no inhibition as compared to that produced by 10% of unheated pH 4.2-soluble protein (compare 4 and 8, Exp. III). The effect of the growth inhibitor is also demonstrated when included in a diet in which casein was the protein component (9, 10, and 11, Exp. III).

The question of the identity or non-identity of the trypsin inhibitor with the growth inhibitor cannot be answered from the present data. The trypsin inhibitor is present in the acid-soluble fraction of soybean proteins, and the 2 may therefore be identical. Since the tryptic inhibitor has recently been isolated in crystalline form by Kunitz,[8] the question can best be answered by inclusion of the isolated substance in the diet. Such studies, together with further characterization of the growth-inhibiting substance, are under way and will be reported at a later date.

Summary. A substance which inhibits the growth-promoting properties of proteins in rats is present in raw soybeans. The substance is non-dialyzable, precipitated by salt, and inactivated by heat. It appears, therefore, to be a protein, which has been shown to be concentrated in the acid-soluble (pH 4.2) fraction of the soybean protein. These results are in agreement with those presented by Ham, Sandstedt, and Mussehl[7] for the chick.

[8] Kunitz, M., *Science*, 1945, **101**, 668.

15354

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and respiratory metabolism have already been reported.[11,12] This investigation presents data on the urinary output and the renal excretion of phosphorus in individuals maintained in the low pressure chamber at altitudes of 8,000 and 10,000 ft. for periods of 10 hours without supplementary oxygen.

Materials and Methods. The detailed procedure for the experiment has been previously described.[12] The data are based on a total of 72 man runs and represent additional measurements taken in the foregoing investigation. Each of the 5 subjects received at least 6 runs at ground level (800 ft.) and a minimum of 5 runs at the 8,000 or 10,000 ft. level. Flights were taken once weekly and alternated between altitude and ground level. Subjects reported to the laboratory in the post-absorptive state. No attempt was made to control the amount nor composition of the meal taken at the regular mess 12-15 hours the evening before. Various metabolic measurements were made in the initial fasting state and all urine was voided at this time. Each subject was then given a standard K-ration breakfast (884 cal.) with 250 cc of water. After consumption of this meal, the chamber was brought to the desired pressure altitude, and the subjects maintained there at rest for 10 hours breathing ambient air and without food. Although water was allowed *ad libitum* in the major experiment, the present data are based on runs during which no water was taken throughout exposure. The control runs were done in the pressure chamber under otherwise identical conditions. Urine collections were made at the end of the first, third, fifth, seventh, and tenth hours of exposure (the "first hour" collection period represents a 2-hour sample, that is, the time from which the initial fasting urine was voided to the end of the first hour of the experiment proper). Urinary phosphorus was determined by the method of Fiske and Subbarow.[13]

Results. Examination of the data in Table I reveals that the total output of urine at both altitudes studied was not appreciably altered from the ground-level condition. The mean hourly secretion of urine (all subjects combined), as averaged for the entire exposure period, was 55 cc at ground level as against 46 cc (2 subjects) and 59 cc at the 8,000 and 10,000 ft. altitudes respectively. In only one individual (G. B.) was the urine volume consistently and significantly increased at altitude (10,000 ft.) The mean hourly secretion in this instance was 57 cc at ground level and 76 cc at altitude, a mean increase of 25.9% at the 10,000 ft. level.

The characteristic feature of urine elimination, as seen in this study, was the considerable degree of variation found in any individual at ground level or altitude. Urine volumes at corresponding time intervals varied widely in successive runs. As little as 200 cc or as much as 1200 cc of urine were voided over the entire 11-hour period on some occasions. The time in exposure at which maximal elimination occurred also differed among subjects. The highest rates of urine output were found within the first half of the exposure period at ground level or altitude, and very likely represented the elimination of the water ingested with the standard breakfast. There was no evidence to indicate that significant differences existed between ground level and altitude as regards urinary output in early as against late exposure. Total urine elimination for the entire experimental period was of the same order of magnitude in all subjects.

The renal excretion of inorganic phosphorus was significantly decreased in all subjects at altitude (Table II). The reduction bore no apparent relationship to urine volume (Table 1). The mean total phosphorus output for the entire exposure period (all subjects combined) was 332 mg (range, 259-392 mg) at ground level, 244 mg (range, 227-261 mg) at 8,000 ft., and 233 mg (range, 195-298 mg) at the 10,000 ft. level. These values represent a mean decrease from the ground level excretion of 29.4% and 22.6% at the higher and lower altitude respectively. Figures for the 8,000 ft. level are based on determinations made in 2 subjects only, in one of

11 D'Angelo, S. A., *Am. J. Phy.*, 1946, **145**, 365.

12 D'Angelo, S. A., *Am. J. Phy.*, in press.

13 Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

TABLE I.
Urinary Output at Ground Level and Altitude with Exposure Time.

Subject	Time (hr)	Ground level (800 ft.) Vol. (cc)	Rate‡ (cc/hr)	8,000 ft. Vol. (cc)	Rate (cc/hr)	10,000 ft. Vol. (cc)	Rate (cc/hr)
	*	†					
	IF-1	123±32		152±33		153±54	
R.B.	1-5	262±43		235±18		302±74	
	5-10	184±25	52	196±44	53	198±40	59
	IF-1	55±27				54±24	
S.K.	1-5	452±112				456±135	
	5-10	188±48	63			164±42	61
	IF-1	86±21		84±17		65±48	
L.K.	1-5	232±22		176±48		254±34	
	5-10	242±52	51	172±50	39	242±64	51
	IF-1	151±49				130±35	
G.B.	1-5	266±50				316±52	
	5-10	206±22	57			394±47	76
	IF-1	74±22				66±18	
C.K.	1-5	237±30				212±40	
	5-10	275±42	53			279±51	51
Combined avg			55		46		60

* Represents a 2-hour interval which includes an hour period at ground level when initial fasting determinations were made and breakfast consumed, and the first hour of the experimental period proper.

† Represents the mean urine volume ± the standard deviation.

‡ Refers to the mean hourly secretion of urine over the 11-hour period of observation.

TABLE II.
Phosphorus Excretion at Ground Level and Altitude with Exposure Time.

Subject	Time (hr)	Ground level P. exer. (mg/hr)	Total P.†	8,000 ft. P. exer. (mg/hr)	Total P.	10,000 ft. P. exer. (mg/hr)	Total P.	% decrease at alt. 8,000	10,000
	IF-1	30.8(2.3)*		29.3(3.6)		25.4(4.3)			
R.B.	1-5	29.4(2.6)		20.0(3.9)		17.7(3.4)			
	5-10	38.4(2.0)	371	19.6(5.1)	227	20.8(2.3)	226	38.9	39.2
	IF-1	7.5(2.0)				10.0(0.8)			
S.K.	1-5	27.9(3.6)				20.1(2.1)			
	5-10	26.5(1.7)	259			20.6(2.3)	203		21.5
	IF-1	18.3(2.9)		28.4(2.6)		16.1(3.5)			
L.K.	1-5	20.3(2.3)		15.1(2.5)		15.5(2.0)			
	5-10	32.1(2.6)	278	28.7(5.6)	261	20.1(1.4)	195	6.3	30.0
	IF-1	25.6(4.1)				22.2(2.5)			
G.B.	1-5	32.3(5.0)				23.0(3.5)			
	5-10	42.3(3.2)	392			32.3(2.9)	298		23.9
	IF-1	15.0(2.1)				12.5(2.2)			
C.K.	1-5	23.6(4.1)				20.5(3.2)			
	5-10	47.4(3.2)	361			27.6(3.3)	245		32.2
Combined avg			332		244		233	22.6	29.4

* Represents the mean rate of phosphorus excretion, and in parentheses, the standard error of the mean.

† Refers to the total phosphorus excretion for the 11-hour period.

whom phosphorus excretion was just slightly diminished.

Excretion rates at ground level increased with exposure time so that the phosphorus content of urine was substantially greater during the last half of the experimental period. This increase with continuing exposure was either lessened or abolished at altitude, the diminution becoming apparent at either altitude level within the first half of the exposure period. Aside from relatively high values of phosphorus excretion during the first-hour interval in one subject, subsequent values at 8,000 ft. approximated those at the higher level and were significantly lower than those at ground level for corresponding time intervals.

Discussion. The results of these experiments indicate that urinary water secretion is not appreciably affected in the resting subject during prolonged exposures at moderately low altitudes. These findings are in accord with those of Bryan and Ricketts[9] at higher altitudes but fail to confirm the results of Armstrong and Heim[8] at the 12,000 ft. level. The former group exposed subjects to altitude pressures of 11,500, 16,600 and 18,000 ft., for 4 to 6 hours, 6 days a week, from 4 to 6 weeks under controlled metabolic conditions. In only one individual was there a significant increase in the volume of urine passed at altitude (16,600 ft.) McFarland and Edwards[7] did describe increased urine elimination in actual flight at altitudes comparable to those in this study. The polyuria, however, was best associated with the nervous tension of piloting the ship. Diet and fluid intake, moreover, were not controlled.

The considerable variation found in the output of urine, despite controlled conditions of food and water intake, indicates the importance in studies such as these of making sufficient measurements over reasonably long periods of observation. In this regard, it must be stated that the 24-hour urine volume was not determined, nor was the fluid intake for each subject known prior to the experimental period. It is believed, however, that determination of the urine volume over an 11-hour period should reveal any existing effect of moderately low altitude on urinary output. There was no indication that at altitude an initial polyuria occurred which was subsequently compensated for by oliguria, as may occur in the rat. Silvette[14] described a marked and sustained polyuria in rats given daily 3-hour exposures at 15,000 and 25,000 ft., whereas, Swann and colleagues[15,16] found no appreciable change in urine output, despite negative water balances, in longer exposures (6-24 hours) at an 18,000 ft. level.

It becomes evident as studies on anoxia continue that physiological changes begin to occur in the body at altitudes lower than previously thought. The present study indicates that the renal excretion of phosphorus may be affected at altitudes as low as 8,000 ft. We can advance no explanation for this effect at present. Since it is elicited at ground level breathing appropriate nitrogen-oxygen mixtures, it is more clearly attributable to the reduced oxygen tension than the reduced barometric pressure *per se*.[6] In the experiments of Bryan and Ricketts[9] the excretion of phosphorus was definitely decreased in the urine passed at altitude, but the 24-hour output was unchanged. Urinary calcium decreased also, but in a manner unrelated to phosphorus.

Whether or not the diminished excretion of phosphorus is primarily a reflection of an acid-base shift in the anoxic organism, or whether it fundamentally reflects some change in the intermediary metabolism is not clear. Alteration in phosphate concentration of the body fluids in acidotic and alkalotic states is well established. The intimate relationship between phosphorus and carbohydrate metabolism is also well known, and needs no elaboration here. In this study, the phosphorus excretion could not be correlated directly with changes in the respiratory metabolism.[12] Although phosphorus output was decreased to a greater extent in sub-

[14] Silvette, H., *Am. J. Phy.*, 1943-44, **140**, 374.

[15] Swann, H. G., Collings, W. D., Cline, J. K., and Dernehl, C. U., *Science*, 1942, **96**, 588.

[16] Collings, W. D., Swann, H. G., Dernehl, C. U., and Cline, J. K., *Fed. Proc.*, 1943, **2**, 7.

jects showing respiratory alkalosis, it appears significant that in the one subject (S. K.) displaying no change in respiration urine phosphorus was appreciably diminished. The changes in the renal excretion of inorganic phosphorus may well reflect some change in the carbohydrate metabolism. Leipert and Kellersman[17] found in human subjects at 5500-6200 meters that a decrease in the phosphorus of blood and urine was compensated for by an increase of phosphate ester in the blood, an effect further augmented by glucose. Similar changes in rats could be correlated with greatly increased liver glycogen. Whether this relationship existed in the present experiments at moderately low altitudes cannot be stated. In this connection, it was found that the blood sugar level remained unaffected at the 8,000 and 10,000 ft. levels.[11]

Conclusions. 1. No appreciable change in total urine output from ground level values was found to occur in human subjects during prolonged exposures to simulated altitudes of 8,000 and 10,000 ft. under conditions of restricted food and water intake.

2. The renal excretion of phosphorus was significantly decreased at altitude.

3. These experiments indicate that alteration in the mineral metabolism may occur in the unacclimatized human being at altitudes as low as 8,000 ft.

[17] Leipert, T., and Kellersman, E., *Z. Physiol. Chem.*, 1942, **276**, 214.

15355

Blood Agglutinins in Filariasis.

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From the United States Naval Medical Research Unit No. 2.†

In a recent study of filariasis on Okinawa,[1] data on the frequency distribution of the A, B blood groups were collected. Certain differences in the frequencies of the blood groups, in comparison to the systematic variation in the general population, were noted to occur in filarial patients who had circulating microfilariae; these differences warrant a brief note at this time.

The blood-group percentages of 1,000 persons from the villages of Fukeyama, Jinusza and Soke[2] (approximately 5% of the population) were compared with the frequency distribution of the A, B blood groups in 180 persons, from the same villages, who had circulating microfilariae. The degree of microfilaremia was determined by dilution counting[1] and its relationship to the blood group percentages was also studied. As noted in Table I, the incidence of filaremia is greater in persons whose sera contain no natural antibody to group A red blood cells. When the data were examined without reference to the variables that influence the degree of filaremia, it was found that persons whose blood did not contain α-agglutinins had, as a rule, higher microfilarial counts. High α- and β-isoagglutinin titers (800-1600) were observed in the sera of many filarial patients, even in those who did not have microfilaremia. In several instances, the sera contained ag-

* The writer is indebted to Comdr. Harold Fink, MC, USNR, who rechecked the blood group typings on all of the filarial patients, and who is responsible for the compilation of the A, B blood group percentages of the Okinawans.

† The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse the views expressed in this paper.

[1] Franks, M. B., Chenoweth, B. M., Jr., and Stoll, N. R., *Am. J. Trop. Med.*, 1946, in press.

[2] Fink, Harold, report to be submitted.

TABLE I.
Frequencies of the A, B Blood Groups in Persons with Microfilaremia Compared with the Systematic Variations in the Frequencies of the Blood Groups on Okinawa.

	A	AB	O	B
% in 1,000 Okinawans*	40.8	8.3	31.8	19.1
% in 180 persons with filaremia	52.0	10.3	25.9	11.8
†Statistical significance of differences	Significant	Not significant	Possibly significant	Significant

* Comdr. Fink has since done 500 additional typings from these villages without any appreciable change in percentages given here.

† The odds against the occurrence of the differences in the ratios arising by chance alone are approximately 40 to 1 for A, 1 to 1 for AB, 10 to 1 for O, and 140 to 1 for B.

glutinins active for the agglutinogens present in their cells. One group A and 4 group AB patients gave anomalous reactions of this type. The α-agglutinins were found in 4 of these cases and were present in low titer.

The possible physiological function of the blood group substance is not known. The serological relationship of A substance to some bacterial carbohydrates suggests that the blood group substances may have an immunological role. However, there is no experimental evidence that one blood group or type enjoys any advantage over the other in this respect.

Oliver-Gonzalez[3,4] has been able to demonstrate that the polysaccharides of several helminths (originally isolated by Campbell from *Ascaris lumbricoides*) inhibits the α- and β-agglutinins of human sera. Although a similar polysaccharide has not been isolated in the filarid, it is possible that such a substance related to the blood group substances exists and might account for the relationship noted above in the filarial patients. In another experiment, Oliver-Gonzalez[5] reported extraordinarily high isoagglutinin titers in malaria patients with blackwater fever, suggesting that the malaria parasite contains blood group substances or related antigens. Heidelberger and Mayer[6] recently noted that persons of blood group O or B, who were vaccinated with malaria parasites, were more likely to cease relapsing than persons of blood group A or AB. They also demonstrated significant rises in agglutinin titers of the blood group O or B volunteers whom they injected with malaria vaccine prepared from infected O or B blood. Control subjects injected with normal human blood group O stromata showed no changes in their α-agglutinin titers.

The data here are not detailed enough to determine the immunological significance of the presence of autoagglutinins in the 5 sera from filarial patients. An attractive hypothesis is one that provides for the production of the agglutinin as the result of the stimulation by a blood group-like substance in the filarid. However, our present knowledge lends no support to such a view. The data are difficult to assess, but the suggestion that there might be a relationship between the natural antibodies of the red blood cells and the antibodies that have a selective action on an organism, indicates the desirability of adding information along these lines.

3 Oliver-Gonzalez, Jose, *J. Infect. Dis.*, 1944, 74, 81.

4 Oliver-Gonzalez, Jose, and Torregrosa, M. V., *J. Infect. Dis.*, 1944, 74, 173.

5 Oliver-Gonzalez, Jose, and Montilla, E., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 169.

6 Heidelberger, Michael, and Mayer, Manfred, *N. R. C. Bulletin on Malaria Research*, September 20, 1944.

15356

Toxicity of Nicotinic Acid and Some of Its Derivatives.

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Handler and Dann[1] observed that the incorporation of large amounts of nicotinamide into the diet caused a decreased growth rate in young rats whereas nicotinic acid and trigonelline had little effect. It was concluded that the decreased growth rate was due to the depletion of the stores of methionine (which was deemed essential for the methylation of nicotinamide) since in the process of detoxication nicotinamide was methylated and excreted as what appeared to be trigonelline. Later investigations[2] indicate that the principal detoxication product is more probably nicotinamide methochloride (N^1-methylnicotinamide).

The marked toxicity of nicotinamide when administered subcutaneously leads one to suspect that the molecule itself exerts a toxic action which is independent of the effect of the compound on the depletion of the stores of "methyl donors." To determine the influence of molecular configuration on toxicity highly purified preparations of pyridine, nicotinic acid (as the sodium salt), nicotinamide, coramine (N,N-diethylnicotinamide), and the respective methyl derivatives of these compounds were administered subcutaneously to young rats of both sexes weighing between 50 and 100 g. The dose needed to kill one-half of the animals in each group was determined. The results appear in Table I.

TABLE I.
Relative Toxicity of Some Derivatives of Pyridine (Subcutaneous Injection).

	LD_{50}* g/kilo	Relative-order of toxicity
Pyridine	1.00	5.00
Pyridine methochloride	0.28	17.9
Nicotinic acid	5.0	1.0
Trigonelline	5.0	1.0
Nicotinamide	1.68	3.0
Nicotinamide methochloride	2.40	2.08
Coramine	0.24	20.8
Coramine methochloride	1.90	2.63

* LD_{50} = dose needed to kill one-half of the animals.

Methylation decreases the toxicity of coramine and nicotinamide, increases the toxicity of pyridine and has little or no apparent influence on the toxicity of nicotinic acid. It is certain that methylation is not the only factor concerned in the toxicity of these compounds since nicotinamide methochloride is more toxic than nicotinic acid. If pyridine is methylated in the rat as it is in the dog[3] this is an example of the conversion of a toxic compound into one which is even more toxic in the process of "detoxication."

Nicotinamide, coramine and the methyl derivatives of these compounds cause paralysis of the respiratory center when administered in large doses. The injection of pyridine and pyridine methochloride in amounts slightly below the lethal dose produced a deep anesthesia of about 2 hours' duration. These compounds had no apparent effect on respiration. Trigonelline and nicotinic acid have such a low order of toxicity that an accurate estimation is impossible since in the massive doses employed the principal effect may be due to the administration of large amounts of hypertonic solutions.

With regard to the effect of the configura-

[1] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 357.

[2] Huff, J. W., and Perlzweig, W. A., *Science*, 1943, **97**, 538; Ellinger, P., and Coulson, R. A., *Nature*, London, 1943, **152**, 383.

[3] His, W., *Arch. exp. Path. u. Pharm.*, 1887, **22**, 253.

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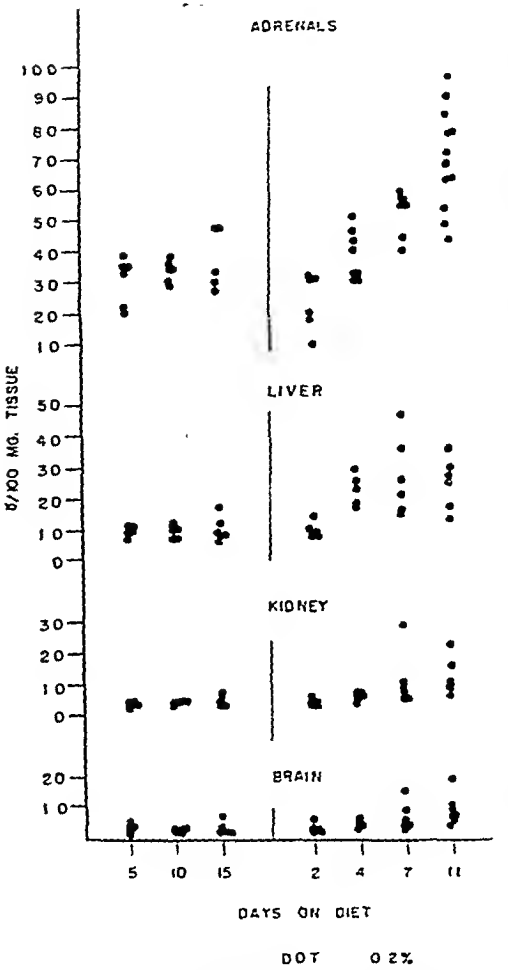


Fig. 1.
Distribution of DDT in brain, kidney, liver and adrenals of rats after ingesting diets containing 0.1 and 0.2% DDT.

DDT was determined by the Schechter-Haller[7] colorimetric method. Both adrenals and aliquots of kidney, liver and brain extracts were used for analyses. The Evelyn photometer with filter No. 600 was used. Standard solutions of DDT were analyzed with each set of determinations to serve as checks for accuracy of the analysis.

Results. The results of the analyses are shown in Fig. 1. The tissues of animals fed the diet containing 0.1% DDT store small amounts of the agent and the concentrations of DDT remain constant during the 5-15-day period of observation. The animals gained weight despite the characteristic tremors while on this experimental diet and no deaths occurred during the period of observation.

The DDT concentrations of the brain and kidney of rats on the diet containing 0.2% DDT increase slightly on the 7th and 11th days. It is surprising that the brain, which is rich in total lipids, stores only a small amount of DDT. The concentration in the liver appears to reach a maximum on the 4th day. There is a marked and significant increase in the DDT concentration in the adrenals which reaches its maximum on the 11th day. The adrenals are comparatively rich in lipids, but no data concerning the changes in the amount and character of these lipids are available. All animals lost weight and approximately half died during the first 9 days.

Summary. The DDT contents of the liver, kidney, brain and adrenals of rats fed diets containing 0.1% and 0.2% DDT were determined at various intervals.

The amounts of this agent stored in the respective tissues reaches a maximum within a few days and remains at a constant level in animals fed 0.1% DDT. The greatest concentration was noted in the adrenals.

The concentrations of DDT in the brain, liver and kidney of rats fed 0.2% DDT increase slightly and progressively with time. The concentration of this material is markedly increased in the adrenal at the time when the animals are severely intoxicated.

[7] Schechter, M. S., Soloway, S. B., Hayes, R. A., and Haller, H. L., *Ind. and Eng. Chem., Analyt. Ed.*, 1945, 17, 704.

tion on the β position of pyridine it is evident that the presence of the carboxyl group decreases the toxicity of the molecule. Conversion of this group to the simple amide markedly increases toxicity; the presence of a substituted amide on this position increases the toxicity so that it exceeds that of the unsubstituted pyridine. The high toxicity of coramine suggests the necessity for caution in its administration.

Summary. The relative toxicity of subcutaneous injections of nicotinic acid, nicotinamide, coramine, pyridine and their respective methyl derivatives has been determined. Methylation decreases the toxicity of coramine and nicotinamide, increases the toxicity of pyridine and has little or no apparent influence on the toxicity of nicotinic acid. The toxicity of the non-methylated compounds appears to be due directly to the structure of the compounds rather than to the depletion of the body stores of methyl donors in the process of detoxication.

15357

Distribution of 2,2 (p-Chlorophenyl) 1,1,1 Trichlorethane (DDT) in Tissues of Rats after Its Ingestion.*

STEPHAN LUDEWIG AND ALFRED CHANUTIN. (Introduced by H. E. Jordan).

From the Biochemical Laboratory, University of Virginia, Charlottesville.

The data for the distribution of DDT in tissues of experimental animals fed DDT are limited. Smith and Stohlmann[1,2] analyzed blood, kidney, liver, central nervous-system and bile after acute and chronic poisoning in rabbits and cats by determining organic chloride. The highest DDT concentration was observed in the bile. Laug[3] determined the DDT concentration in tissues of rats fed on diets containing small amounts of DDT for periods varying from 6 months to 2 years. By far, the greatest amount of DDT was found in perirenal fat. In spleen, liver and kidney, the amount of DDT stored appeared to be roughly proportionate to the amount of total ether extractable-material in these organs. Woodward[4] studied the DDT concentrations in tissues of a chronically poisoned dog, monkey, pig and turkey. The highest concentration of DDT was found in fat; the adrenals of the dog and the monkey contained appreciably larger amounts of DDT than the liver, kidney and brain.

The distribution of DDT in the brain, liver, kidney and adrenals of rats fed diets containing 0.1 and 0.2% DDT was determined at frequent intervals.

Methods. Inbred male rats of Wistar stock, 60 to 70 days old, and weighing 150-200 g were used as experimental animals. They were maintained on a stock diet until the experimental diet was fed. The diet contained 20% casein (Labco), 8% crisco, 4% inorganic salt,[5] 65% sucrose, 2% agar, vitamin supplements and 0.1 or 0.2% DDT (M.P. 108.6-109.5°). The animals were fed *ad libitum* and were sacrificed at frequent intervals. After nembutal injection, the animals were exsanguinated and the tissues immediately removed for the DDT determination.

The tissues were prepared for analysis according to Ofner's[6] recommendation and the

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

1 Smith, M. I., and Stohlman, E. F., *Pub. Health Rep.*, 1944, **59**, 984.

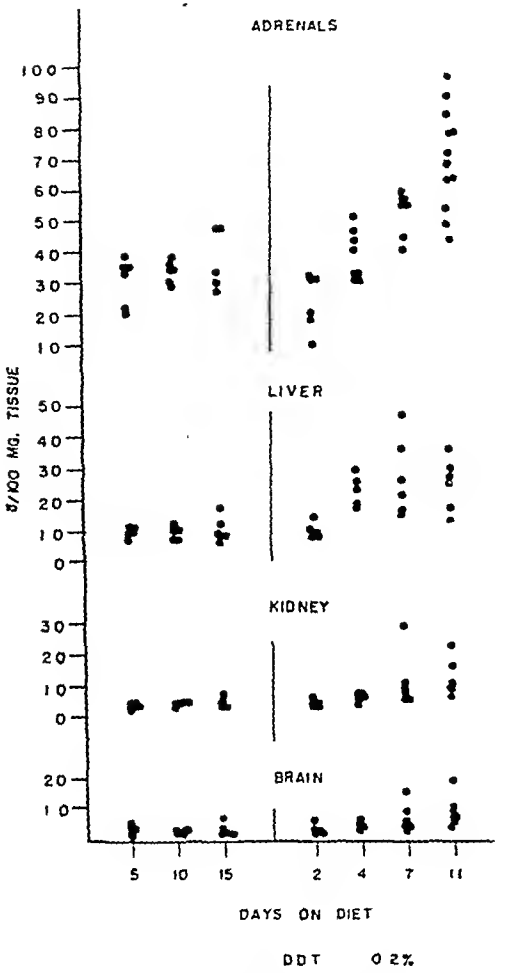
2 Smith, M. I., and Stohlman, E. F., *Pub. Health Rep.*, 1945, **60**, 289.

3 Laug, E. P., personal communication.

4 Woodward, G., personal communication.

5 Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, **15**, 317.

6 OSRD Insect Control Committee, Report No. 65.



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[7] Schechter, M. S., Soloway, S. B., Hayes, R. A., and Haller, H. L., *Ind. and Eng. Chem., Analyt. Ed.*, 1945, **17**, 704.

15358

Age and Species Variation in the Acute Toxicity of Alpha-Naphthyl Thiourea.*

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The general properties of α-naphthyl thiourea (ANTU) have been described in a previous paper,[1] in which it was shown that this substance, besides being an effective poison for the field control of Norway rats, has interesting possibilities as a tool for research because of its actions leading to pulmonary edema, increased lymph flow[2] and other physiological and anatomical effects.[3,4]

The present work was performed to elucidate the practical questions of whether ANTU is toxic to other animals than Norway rats and would therefore present hazards in use, and also whether, as was indicated in field trials, young Norway rats are less susceptible than adults. Differences between males and females were sought at the same time.

Although our primary objectives were practical ones, bearing on the use of ANTU as a rodenticide, the results obtained are presented here because of the many applications of ANTU and other thiourea derivatives to various fields of research.

Age and Sex Variation in Rats—Wild Norway rats were chosen for test animals, because of the large strain differences in response to thiourea poisoning which had been found between wild and laboratory rats,[6] and which it was felt might exist for ANTU as well.

The rats were all wild brown Norways, trapped in the alleys and back yards of Baltimore, Maryland, in special wooden box traps which have been described elsewhere.[6] They were predominantly from residential districts. Care was taken to use only rats trapped in areas where no systematic field poisoning with ANTU had been done. All rats (except some classed as suckling) were held in the laboratory a minimum of 4 days after trapping to allow them to recover from that ordeal and to make sure they were healthy. During this period they were fed Purina fox chow cubes and had access to an ample water supply.

The ANTU was suspended in olive oil and administered by intraperitoneal injection, the dose being adjusted to body weight by giving a volume corresponding to 0.1 cc per 10 g body weight. The rats were not starved; they were, however, injected in the late afternoon, just before the normal feeding time of wild rats.

To restrain the rats for weighing and injecting use was made of the "sock" developed by Emlen.[7] This device made it possible to

* This work was begun under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johns Hopkins University, and completed under a contract between the Chemical Warfare Service and the Johns Hopkins University.

† We are indebted to Dr. E. B. Astwood in Boston for sending us the rats from his colony used in comparative toxicity tests, and to Mr. H. J. Spencer of the Fish and Wildlife Service in Gainesville, Florida, for trapping and shipping to us a number of wild Alexandrine rats.

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2 Drinker, C. K., Pulmonary Edema and Inflammation, Harvard University Press, 1945, 39-43.

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6 Richter, C. P., and Emlen, J. T., Jr., *Public Health Rep.*, 1945, **60**, 1303.

7 Emlen, J. T., Jr., *J. Wildlife Management*, 1944, **8**, 264.

TABLE I.
Age and Sex Variation in Susceptibility of Wild Norway Rats to ANTU Poisoning.

Wt. range (g)		No. rats used Male	Female	Total	Average body wt. (g)	$LD_{50} \pm$ S.E. (mg/kg body wt.)
		Age				
0- 50	suckling	17	7	24	39.3	58±4.4
51-100	weanling	23	17	40	80.7	43±5.7
101-125	young	11	24	35	112.5	22±3.2
126-150	"	15	7	22	140.4	18±4.3
151-200	"	15	14	29	170.1	16±2.7
201-300	young adult	10	26	36	263.1	8.1±0.9
301-400	adult	45	37	82	348.3	7.7±1.0
401-546	"	39	17	56	447.6	6.2±0.6
Total				324		
		Sex				
Above 301	(adult males)	84	—	—		7.0±0.7
" 301	(" females)	—	54	—		7.4±0.9

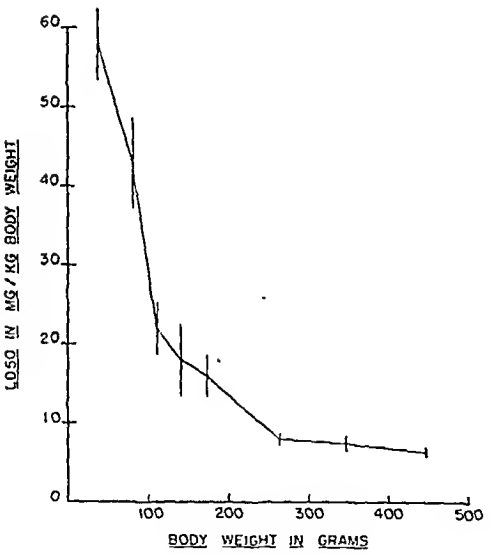

Fig. 1.

Change in acute LD_{50} of ANTU with body weight of wild Norway rats. The LD_{50} for each weight range is plotted against an abscissa representing the average body weight of rats in that range. The standard error of each LD_{50} is indicated by a vertical line of appropriate length.

handle wild rats without anaesthetics in much the same way as laboratory rats.

The assays on rats weighing more than 200 g were carried out over the course of a year and a half (January 1944 to June 1945) with no seasonal variation being noted. The assays on rats below 200 g were performed during April, May and June 1945.

The results obtained with a series of 324 rats are summarized in Table I. The median lethal doses (LD_{50}'s) given in the last column were estimated, together with their standard errors, by the method of Litchfield and Fertig,[8] using logarithmic-probit graph paper.

A comparison of the LD_{50} values given in Table I, and presented graphically in Fig. 1, shows that the resistance to acute ANTU poisoning was greatest in the youngest rats, and then decreased fairly rapidly to level off between 8 and 6 mg/kg for rats weighing 200 g or more. Suckling rats (weighing less than 50 g) and weanling rats (up to 100 g) were respectively about 7 and 5 times as resistant as old rats. Between 100 and 200 g the resistance was still more than twice as great as it was above 200 g. It may be mentioned in this connection that a corresponding (but larger) difference in response to acute poisoning with the parent compound thiourea has been observed between young and adult laboratory rats.[9,5]

The correlation of weight with age, which is indicated by the classifications into suckling (birth to approximately 30 days of age), weanling (30 to 60 days) and adult (over 90 days), is only an estimate, based on observations such as whether the rats were trapped in a group with their mother, were

[8] Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 276.

[9] MacKenzie, J. B., and MacKenzie, C. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 34.

15358

Age and Species Variation in the Acute Toxicity of Alpha-Naphthyl Thiourea.*

SALLY H. DIEKE AND CURT P. RICHTER.†

From the Psychobiological Laboratory, Phipps Psychiatric Clinic, The Johns Hopkins Hospital, Baltimore, Md.

The general properties of α-naphthyl thiourea (ANTU) have been described in a previous paper,[1] in which it was shown that this substance, besides being an effective poison for the field control of Norway rats, has interesting possibilities as a tool for research because of its actions leading to pulmonary edema, increased lymph flow[2] and other physiological and anatomical effects.[3,4]

The present work was performed to elucidate the practical questions of whether ANTU is toxic to other animals than Norway rats and would therefore present hazards in use, and also whether, as was indicated in field trials, young Norway rats are less susceptible than adults. Differences between males and females were sought at the same time.

Although our primary objectives were practical ones, bearing on the use of ANTU as a rodenticide, the results obtained are presented here because of the many applications of ANTU and other thiourea derivatives to various fields of research.

Age and Sex Variation in Rats—Wild Norway rats were chosen for test animals, because of the large strain differences in response to thiourea poisoning which had been found between wild and laboratory rats,[5] and which it was felt might exist for ANTU as well.

The rats were all wild brown Norways, trapped in the alleys and back yards of Baltimore, Maryland, in special wooden box traps which have been described elsewhere.[6] They were predominantly from residential districts. Care was taken to use only rats trapped in areas where no systematic field poisoning with ANTU had been done. All rats (except some classed as suckling) were held in the laboratory a minimum of 4 days after trapping to allow them to recover from that ordeal and to make sure they were healthy. During this period they were fed Purina fox chow cubes and had access to an ample water supply.

The ANTU was suspended in olive oil and administered by intraperitoneal injection, the dose being adjusted to body weight by giving a volume corresponding to 0.1 cc per 10 g body weight. The rats were not starved; they were, however, injected in the late afternoon, just before the normal feeding time of wild rats.

To restrain the rats for weighing and injecting use was made of the "sock" developed by Emlen.[7] This device made it possible to

* This work was begun under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johns Hopkins University, and completed under a contract between the Chemical Warfare Service and the Johns Hopkins University.

† We are indebted to Dr. E. B. Astwood in Boston for sending us the rats from his colony used in comparative toxicity tests, and to Mr. H. J. Spencer of the Fish and Wildlife Service in Gainesville, Florida, for trapping and shipping to us a number of wild Alexandrine rats.

1 Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**, 927.

2 Drinker, C. K., Pulmonary Edema and Inflammation, Harvard University Press, 1945, 39-43.

3 Du Bois, K. P., *Fed. Proc.*, 1946, **5**, 174.

4 McClosky, W. T., and Smith, M. I., *Public Health Rep.*, 1945, **60**, 1101.

5 Dieke, S. H., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1945, **83**, 195.

6 Richter, C. P., and Emlen, J. T., Jr., *Public Health Rep.*, 1945, **60**, 1303.

7 Emlen, J. T., Jr., *J. Wildlife Management*, 1944, **8**, 264.

guinea pig, the cat, and probably the rabbit‡ is followed by the chicken and the Rhesus monkey, both of which were extremely resistant, at least to ANTU given by stomach tube.

It is interesting to note that in contrast to the large variations in the toxicity of thiourea to Norway rats from different sources,[6] little difference was found in the response of the same rats to ANTU. Rats from Dr. Astwood's colony in Harvard (designated Strain II) were only twice as resistant as rats from our colony (Strain I); and hardly differed at all from the wild Norways.

The mode of administering the ANTU did not influence the toxicity in rats, but in other species less ANTU was required to kill by intraperitoneal injection than when given by stomach tube. For instance monkeys died following 200 mg/kg of ANTU given intraperitoneally but withstood 17 times that amount by stomach tube, and dogs likewise showed a several-fold difference. In part this may be ascribed to emesis, which occurred fairly often in dogs, cats, and monkeys, but elimination in this manner before absorption could take place was minimized by starving the animals overnight before they received doses by stomach tube, and withholding food until 6 to 8 hours later.

The effects characteristic of ANTU poisoning in Norway rats, namely pulmonary edema and pleural effusion, were not found in some species even after the administration of fatal doses. Examination of sections from the lungs of Alexandrine rats, guinea pigs, rabbits and monkeys has not revealed edema in anything like the amounts found in the lungs of Norway rats, dogs, mice and cats.[10] It would therefore seem likely that the mechanism of action of ANTU is different in these 2 groups of animals.

Both males and females were used in most species tested (the rabbits and chickens were all females) without any striking sex difference in susceptibility becoming evident. Sex differences cannot be ruled out, however, on the basis of these limited data. An indication that an age difference exists in dogs, comparable to that observed in rats, is found in the fact that 3 puppies survived doses which would have been fatal to adult dogs.

Summary. Marked age variation in the susceptibility of wild Norway rats to acute poisoning with α-naphthyl thiourea has been found, with suckling rats about 7 times as resistant as adults. This resistance was found to decrease with increasing body weight, levelling off for adults at an LD_{50} between 6 and 8 mg/kg body weight. The difference found between adult males and females was within the limits of error and is therefore not considered significant.

Adult animals of other species also varied markedly in susceptibility to acute ANTU poisoning. Norway rats, dogs and mice were killed by amounts less than a hundred milligrams per kilogram. Alexandrine rats, guinea pigs, and cats required several hundred milligrams, while chickens and monkeys survived doses of several grams per kilogram body weight. Large amounts of pulmonary edema were found only in Norway rats, dogs, mice and cats.

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15359

Bactericidal Action of Streptomycin.

DOROTHY HAMRE, GEOFFREY RAKE, AND RICHARD DONOVICK.
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Streptomycin,[1] which inhibits the growth of a variety of gram positive, gram negative and acid fast microorganisms[1-7] has been found by Waksman and Reilly[8] to be bactericidal for *Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris*. According to Wallace

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wild	"	(See Table I)	
"	s.t.	50	6.9±0.5
Dog *(Canis familiaris)*	i.p.	9	below 16
"	s.t.	7	38 (20-50)‡
Mouse *(Mus musculus)* albino	i.p.	19	56±4
Alexandrine rat *(R. rattus subsp.)*	"	14	250 (75-450)
Guinea pig *(Cavia cobaya)*	"	11	350 (300-400)
Rabbit *(Oryctolagus cuniculus)*	", s.t.	6	above 400
Cat *(Felis libyca domestica)*	s.t.	10	500 (75-1000)
Chicken *(Gallus bankiva)*			
Barred Plymouth Rock pullets	i.p.	4	2500 (?-5000)
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Monkey *(Macaca mulatta)*	i.p.	6	175 (150-200)
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‡ Three puppies given ANTU by stomach tube survived doses up to 75 mg/kg.

small but trapped alone and able to fend for themselves, or afforded obvious external indications of maturity such as an open vagina or large descended testes. It is moderately certain that no rat weighing less than 100 g was sexually mature, while all those weighing over 200 g were indubitably adults. The age of puberty thus fell in the weight range of 100 to 200 g; more precisely, in the range 100-150 g for females and 150-200 g for males. This last statement is based upon observations made on 11 female and 5 male wild Norway rats that were raised from birth in the laboratory, which indicated that around puberty the males of a given age are heavier than the females: between 60 and 90 days old these females weighed 100-150 g and the males 150-200 g, with considerable individual variation being found within these limits, particularly between rats of different litters. (In laboratory Norways from our colony the sex difference in weight is even larger, as 60-day-old males often weigh more than 200 g while females of that age rarely weigh more than 140 g.)

In the series of rats receiving ANTU the vagina was open in about half the females with weights between 100 and 125 g, and in all the females weighing more than 150 g, which agrees well with the above statement. No definite difference in resistance to ANTU poisoning was found between individual females with open vaginas and others in the same weight range with closed vaginas, but this external sign is admittedly not the best criterion of sexual maturity. It is therefore not yet clear whether the levelling off of the LD_{50} which occurred after puberty is directly related to sexual maturity or is rather an associated phenomenon dependent on general growth factors.

Separating the 138 rats weighing more than 300 g into males and females led to respective LD_{50} values of 7.0 and 7.4 mg/kg (Table I). Thus adult females were little if any more resistant than males, indicating the absence of a marked sex variation in response to acute ANTU poisoning.

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0				6430
2 hr	17.6	>3000	46300	84600
3 "	0.8	2120	127000	212000
4 "	0.1	154	443000	836000
23 "	820000.	865000	481000	1,150000
	0.75% tryptone broth Units per ml pure streptomycin M208E			
	1.0	0.5	0.25	0
0				6760
20 min	4000.	7000.	7360.	6800
40 "	7.16	3760.	8030.	9700
1 hr	0.01	50.3	>3000.	16200
2 "	0.00	0.04	>3.0	75300
3 "	0.00	0.01	0.08	195000
24 "	0.00	122000.	109000.	753000
0				7250
1 hr		12.0	4580.	21900
2 "		0.03	1.18	67400
3 "		0.02	0.00	144000
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effect of 0.25 unit per ml in broth after 12 hours incubation grew in plates containing 4.9 units per ml, but none grew on plates containing 49 units per ml. Apparently, then, this resistance which was acquired by this organism, or was a result of selection, is not of a high order of magnitude.

To determine whether or not this resistance was a temporary characteristic, transplants were made in the third experiment from the tube containing 0.25 unit per ml in broth after 0 hours and 16 hours incubation, to tubes containing 0.0 unit, 0.07 unit, 0.35 and 1.75 units of streptomycin per ml of tryptone broth. Serial subcultures were made after 24 hours incubation from the tube containing no streptomycin to tubes with and without streptomycin. The results of these experiments are given in Fig. 2 Three things are apparent: (1) After 16 hours incubation in broth containing 0.25 unit per ml, the organisms grew in broth containing 5 times as much streptomycin as the minimal inhibiting dose for the same culture after 0 hours. This was to be expected, since at that time the plate count in agar containing 4.9 units of streptomycin per ml was almost equal to that in agar without streptomycin (Fig. 1). (2) The amount of streptomycin in broth to which these organisms were resistant after 16 hours incubation was much less than that in agar (0.35 unit per ml compared to 4.9 units per ml, Fig. 1 and Fig. 2); (3) after 3 subcultures in the absence of streptomycin, the 2 cultures retained their respective characteristics, an indication that a persistent change in the resistance of the organism had occurred.

Some evidence was also obtained for the appearance of resistance in *Klebsiella pneumoniae* by repeated subculturing in tryptone broth containing streptomycin. After 2 serial subcultures in 0.35 unit of streptomycin per ml, the resistance of the organisms taken originally from broth containing 0.25 unit per ml after 16 hours incubation rose from 0.35 unit per ml to 1.75 units per ml. (Fig. 2). However, this 5-fold increase in resistance is slight in comparison with the increase in resistance of gonococci and meningococci from about 40 units per ml to 75,000 units per ml which Miller and Bohn-

and co-workers,[9] the bactericidal action of streptomycin on *Staphylococcus aureus* and *Eberthella typhosum* was influenced by the culture medium. Donovick and Rake[10] found that 0.056 units per ml of streptomycin inhibited the growth of 1000 cells per ml of *Klebsiella pneumoniae* in 0.75% tryptone broth and that the minimal inhibiting dose varied with the lot and the concentration of tryptone. The present study of the bactericidal action of streptomycin was made on *K. pneumoniae,** which is used in this laboratory for the bio-assay of streptomycin.[11]

In order to follow the bactericidal action of streptomycin on this organism, 6 ml of a diluted 6-hour culture containing about 5 million cells per ml were mixed with 0.1 ml of streptomycin and incubated at 37°C. Plate counts, made at intervals in 2% tryptone 0.2% glucose agar, are given in Table I. All figures for bacterial counts are averages of 3 plates.

Comparison of the results in yeast beef broth with those in tryptone broth illustrates the effect of culture medium on the activity of streptomycin. While 2 units per ml in yeast beef broth retarded growth slightly, 1 unit per ml in tryptone broth produced a 500,000-fold reduction in count in one hour. Although different preparations of streptomycin were used, this could not account for these results, because repeated experiments in the same medium have failed to show any difference between the activity of pure and impure streptomycin.

The bactericidal effect of 1.0 unit of streptomycin per ml in tryptone broth was evident after 20 minutes incubation. On the other hand, 0.25 unit per ml caused no reduction in count during the first hour of incubation, but, following this induction period, the count dropped 60,000-fold in 3 hours, and after 24 hours incubation, rose to about one-seventh of that in the control tube. This rise in count, which also occurred in tryptone broth containing 0.5 unit per ml of streptomycin, was contrary to expectations because 1000 cells per ml in tryptone broth are inhibited by 0.056 unit per ml. Two possible explanations for the rise in count are: (1) that during the 3-hour incubation period in the presence of large numbers of cells, the streptomycin was used up, so that the concentration fell below the minimal inhibitory level; or (2) that the cells surviving at the end of 3 hours incubation with streptomycin were more resistant to its action. Experiments were set up to test the latter hypothesis. Plate counts were made in 0.75% tryptone agar with and without streptomycin. Results of these experiments are given in Fig. 1.

Although there were a few cells in the control tube resistant to 1.0 units and 3.0 units of streptomycin, the proportion of resistant to total cells in this tube remained small throughout the experiment. However, in the tube containing 0.25 unit of streptomycin per ml, the number of resistant cells in comparison to total cells, which was small after 3 hours incubation, became almost equal after 6 hours incubation and remained so through 12 and 16 hours incubation. (Exp. 2, Fig. 1). In a third experiment, when the concentration of streptomycin in the agar was increased to 4.9 and 49 units per ml, almost all the organisms surviving the bactericidal

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1 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

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9 Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 127.

10 Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

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11 Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

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	1.0	0.5	0.25	0
0				6760
20 min	4000.	7000.	7360.	6800
40 "	7.16	3760.	8030.	9700
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To determine whether or not this resistance was a temporary characteristic, transplants were made in the third experiment from the tube containing 0.25 unit per ml in broth after 0 hours and 16 hours incubation, to tubes containing 0.0 unit, 0.07 unit, 0.35 and 1.75 units of streptomycin per ml of tryptone broth. Serial subcultures were made after 24 hours incubation from the tube containing no streptomycin to tubes with and without streptomycin. The results of these experiments are given in Fig. 2 Three things are apparent: (1) After 16 hours incubation in broth containing 0.25 unit per ml, the organisms grew in broth containing 5 times as much streptomycin as the minimal inhibiting dose for the same culture after 0 hours. This was to be expected, since at that time the plate count in agar containing 4.9 units of streptomycin per ml was almost equal to that in agar without streptomycin (Fig. 1). (2) The amount of streptomycin in broth to which these organisms were resistant after 16 hours incubation was much less than that in agar (0.35 unit per ml compared to 4.9 units per ml, Fig. 1 and Fig. 2); (3) after 3 subcultures in the absence of streptomycin, the 2 cultures retained their respective characteristics, an indication that a persistent change in the resistance of the organism had occurred.

Some evidence was also obtained for the appearance of resistance in *Klebsiella pneumoniae* by repeated subculturing in tryptone broth containing streptomycin. After 2 serial subcultures in 0.35 unit of streptomycin per ml, the resistance of the organisms taken originally from broth containing 0.25 unit per ml after 16 hours incubation rose from 0.35 unit per ml to 1.75 units per ml. (Fig. 2). However, this 5-fold increase in resistance is slight in comparison with the increase in resistance of gonococci and meningococci from about 40 units per ml to 75,000 units per ml which Miller and Bohn-

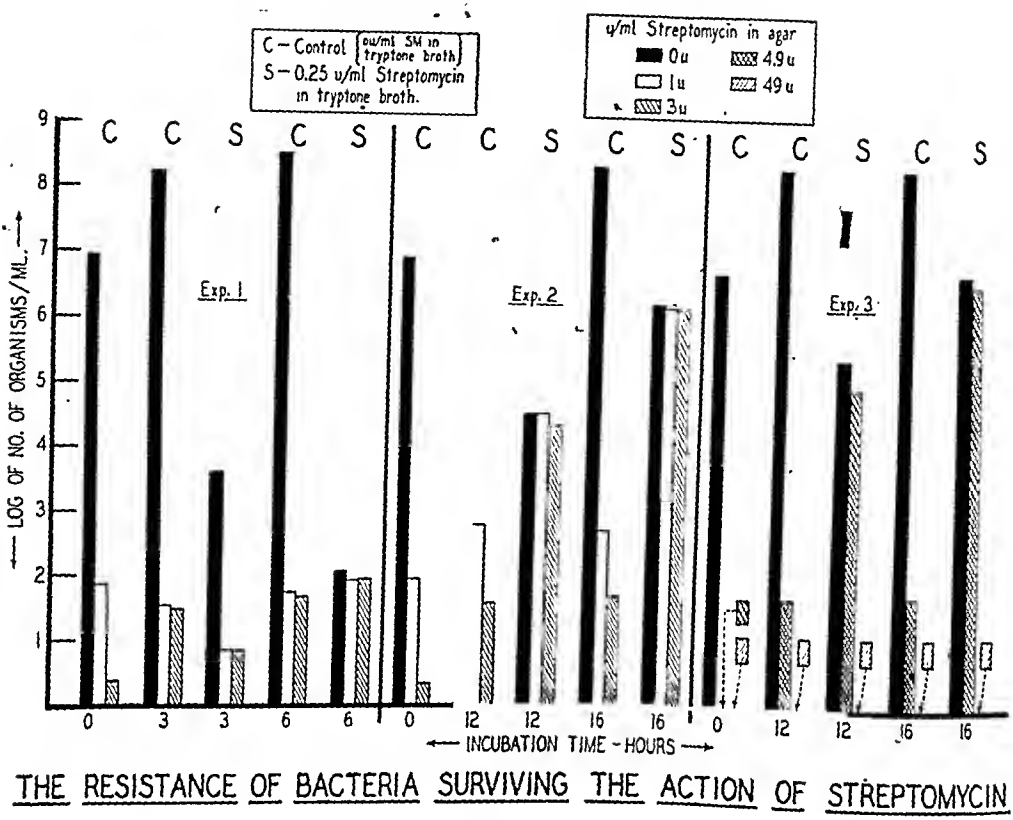

Fig. 1.

TABLE II.
Bactericidal Action of Streptomycin on Washed Cultures of *Klebsiella pneumoniae*.
(No. of Organisms per ml × 1000).

Incubation time	Units per ml pure streptomycin M208E			
	30	15	7.5	0
0				5760
20 min	1100.	5300.	5530.	6100
40 "	3.55	616.	5000.	5730
1 hr	.02	10.4	1983.	5430
2 "	<.01	.023	12.9	4900
3 "	<.01	.013	.066	7300
	15	7.5	3.75	0
0				5700
20 min	5600.	6100.	6100	5230
40 "	2590.	4230.	5830	5430
1 hr	220.	3360.	5600	5560
2 "	.00	108.	2050	7960
3 "	.00	.00	172	7800
		3.78	1.89	0
0				4530
2 hr		5260	5860	5700
4 "		3240	6800	7600
6 "		443	6130	8130

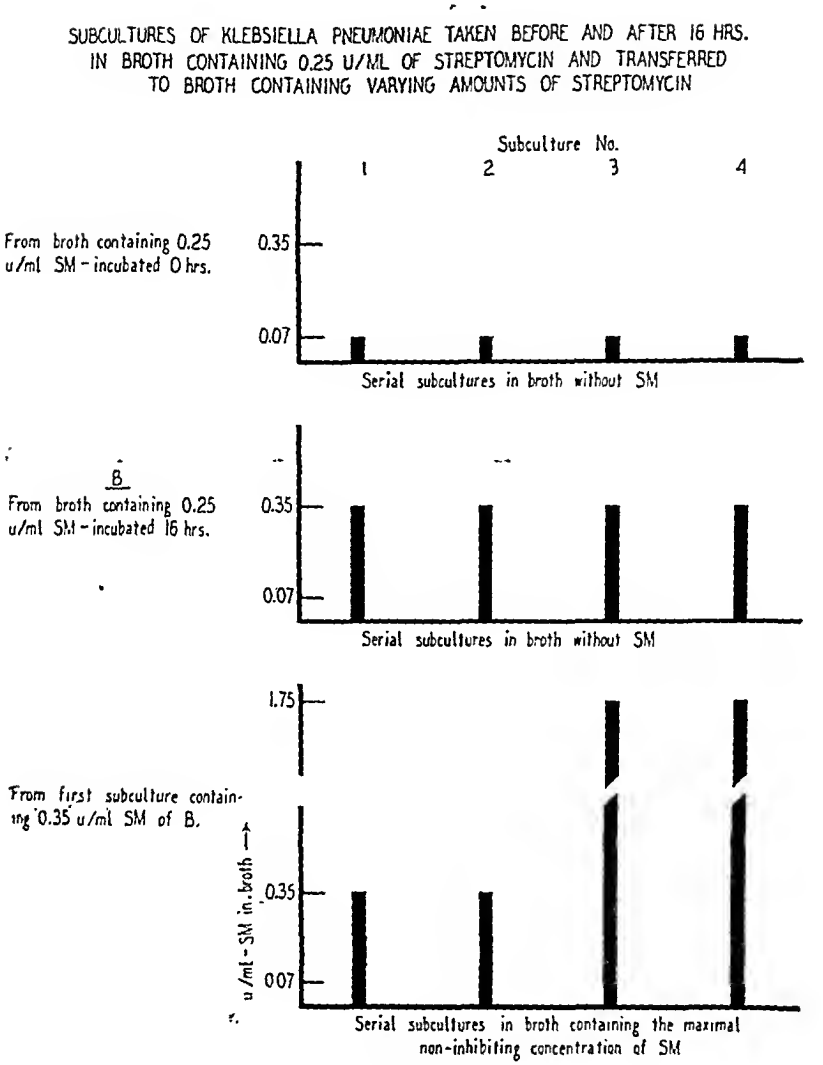

Fig. 2.

hoff[12] were able to obtain by serial transfer on agar containing streptomycin.

The bactericidal action of streptomycin on non-multiplying cultures of *K. pneumoniae* was next investigated. A 6-hour culture was washed 3 times by centrifuging with Ringer's solution, resuspended in Ringer's solution to give approximately 10 million cells per ml and mixed with an equal volume of streptomycin in M/15 phosphate buffer pH 6.8. During incubation at 37°C plate counts were made in 2% tryptone 0.2% glucose agar. Results of average counts of 3 plates are given in Table II.

Thirty units per ml reduced the number of viable cells greater than 5-fold after 20 minutes incubation, 3.75 units per ml caused a slight drop in count after 3 to 6 hours incubation, but 1.89 units per ml had no effect. These results indicate that the mode of action of streptomycin differs from that of penicillin, which had no action on

[12] Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1945, **130**, 485.

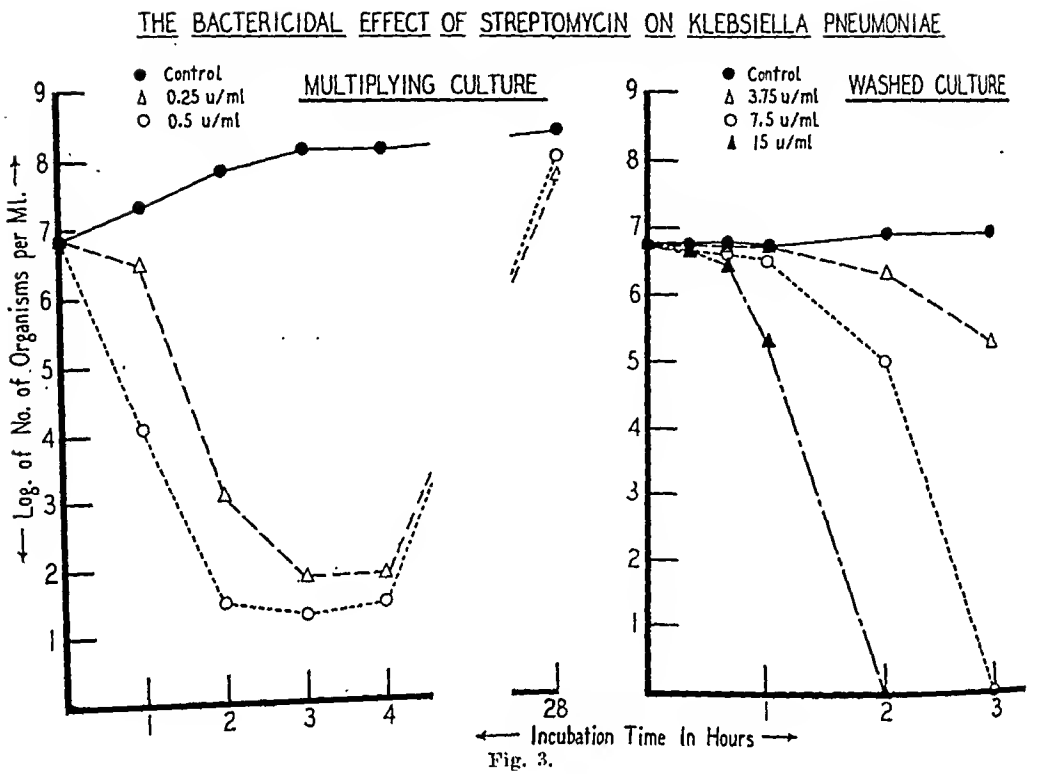

Fig. 3.

a washed culture of *Staphylococcus aureus*, Heatley strain, even at 1000 units per ml.[13]

Because the amount of streptomycin required to produce a drastic drop in the number of viable cells in a washed, non-multiplying culture is much greater than that required for a multiplying culture (Fig. 3), it seems probable that the mode of action of streptomycin is different for washed, non-multiplying bacteria.

It will be recalled that with multiplying cultures almost all the organisms surviving 6 hours incubation with 0.25 unit of streptomycin per ml were more resistant to streptomycin. In experiments with washed cultures, incubated with 7.5 units per ml and plated in agar containing 1.0 and 3.0 units of streptomycin per ml, no evidence was obtained to indicate that the organisms surviving 3 hours or 5 hours incubation were more resistant than the control. This also favors the theory that the mode of action of streptomycin on washed organisms is different from that on multiplying cultures.

In view of the bactericidal action of streptomycin on washed cultures of *K. pneumoniae*, it was of interest to determine whether or not streptomycin had any action on washed spores of a susceptible species. It was found that 43,100 units per ml in buffered Ringer's solution had no effect during 6 hours or 24 hours incubation with washed spores of an unidentified gram positive bacillus (*Bacillus sp.* No. 290). Multiplying cultures of this organism were very sensitive to streptomycin, being inhibited by as little as 0.013 unit per ml in tryptone broth containing 1500 cells per ml.

Summary. Streptomycin was bactericidal for both multiplying and non-multiplying cultures of *K. pneumoniae*, but not for washed spores of *Bacillus sp.* No. 290. Organisms surviving the bactericidal effect of 0.25 unit of

[13] Todd, E. W., *Lancet*, 1945, 1, 74.

streptomycin per ml after 6 hours incubation were more resistant to its action. This resistance persisted through 4 subcultures in the absence of streptomycin and could be increased 5-fold by serial transfers in broth containing streptomycin. Washed organisms surviving after 5 hours incubation with 7.5 units of streptomycin per ml were not more resistant.

15360

A Procedure for Testing Sterility of Concentrated Streptomycin Solutions.

GEOFFREY RAKE AND RICHARD DONOVICK.
From the Squibb Institute for Medical Research, New Brunswick, N. J.

Since the use of streptomycin as a therapeutic agent is rapidly increasing it is important to be able to test the sterility of highly concentrated solutions of this antibiotic. In such sterility tests it is, of course, a prerequisite that the sample of streptomycin taken be of sufficient volume to be representative of the lot of streptomycin involved. As the bacteriostatic levels of streptomycin, at least in the case of many organisms, differ greatly from bactericidal concentrations[1] the addition of such an adequate sample of streptomycin to the sterility test broth may result in inhibition of growth of any viable cells which may be present. Hence there is need for a method of inactivating streptomycin without destroying any living organisms present.

In order to test the sterility of streptomycin solutions in this laboratory, a procedure has been devised which incorporates the inactivation of streptomycin with semicarbazide[2,4] and the use, as the culture medium, of thioglycolate broth which interferes with streptomycin activity.[3]

Of the carbonyl reagents which inactivate streptomycin, semicarbazide was chosen because it is one of the least bacteriostatic members of this group (Table I) and is readily soluble in water. Thiosemicarbazide is also relatively low in bacteriostatic activity but is much less soluble.

TABLE I.
Comparative Sensitivity of Various Organisms to Streptomycin and to Certain Carbonyl Reagents.

Organism	Growth inhibiting concentration in 1% tryptone broth					
	Streptomycin mg/ml	HA-HCl mg/ml	HZ-H_2O mg/ml	SC-HCl mg/ml	TSC mg/ml	Ratio SC-HCl/streptomycin
K. pneumoniae (A.T.C.C. No. 9997)	0.000055*	0.0088	0.0068	0.073	0.116	1325
E. coli No. 33	0.00012*	0.016	0.010	0.064	0.088	533
Staph. aureus (Heatley)	0.000049*	0.018	0.017	>0.54	0.017	>11000
B. subtilis No. 558	0.000056*	0.012	0.012	0.48	0.23	8570
Bacillus sp. No. 290	0.000013*	0.010	0.0073	0.089	0.15	6840

* Based on pure streptomycin base as having 1000 units per mg.
HA-HCl = hydroxylamine hydrochloride.
HZ-H_2O = hydrazine hydrate.
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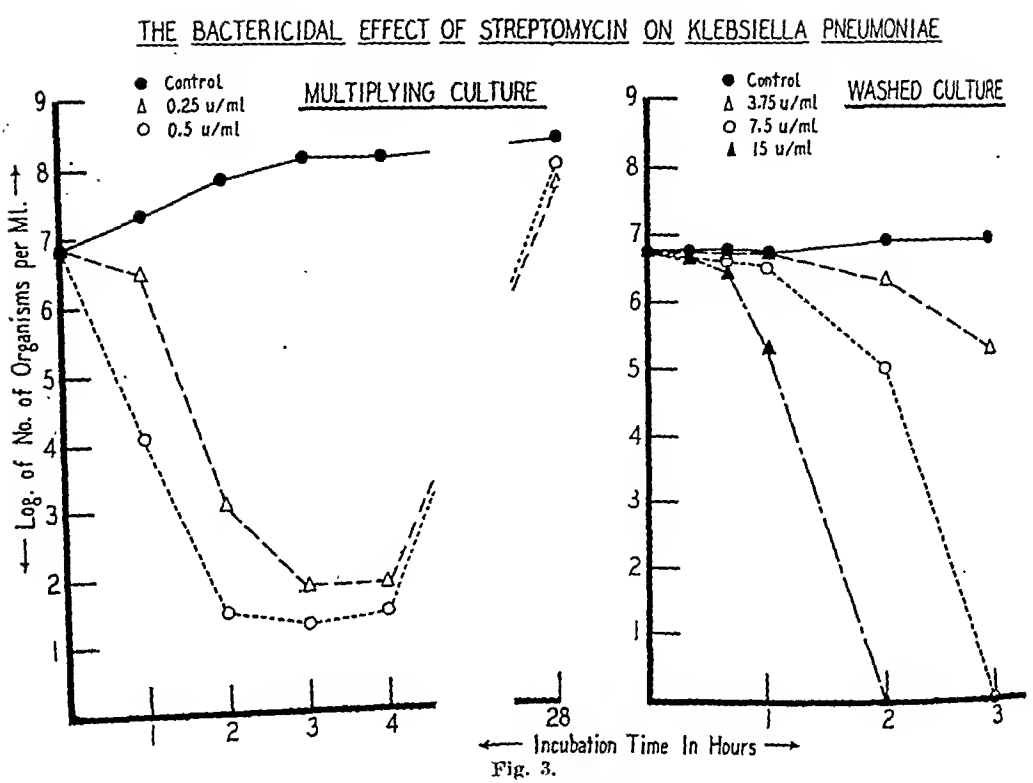
[1] Hamre, D., Rake, G., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 25.

[2] Brink, N. G., Kuehl, F. A., and Folkers, K., *Science*, 1945, **102**, 506.

[3] Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

[4] Donovick, R., Rake, G., and Fried, J., *J. Biol. Chem.*, in press.



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Method. Materials required for inactivation of streptomycin solution containing 30,000 units per ml ± 10%:

1. Semicarbazide-hydrochloride solution: ca. 0.06 g per ml. Sterilization through U. F. fritted glass filter is the preferred method. The sterile solution is stored at 4°C.

2. Potassium acetate solution: ca. 0.05 g per ml. Solution is sterilized by autoclaving, 10 lb pressure for 10 minutes.

Procedure for inactivation of streptomycin. To each ml of streptomycin solution to be inactivated is added 1 ml of the potassium acetate solution and then 1 ml of semicarbazide-hydrochloride solution, observing aseptic precautions. The mixture is incubated at ca. 25°C (room temperature) for 24 hours.

After room temperature incubation, the inactivation mixture is tested for sterility in freshly prepared thioglycolate broth, using at least 30 ml of broth for each ml of inactivation mixture to be tested. The inoculated broth is incubated at 37°C for at least 24 hours.

Discussion. It has been shown that 2 γ of semicarbazide hydrochloride per γ (or unit) of streptomycin will cause 98% inactivation at pH 5.3.[4] Since the semicarbazide-hydrochloride solution is extremely low in pH, potassium acetate is first added to the streptomycin solution to bring the final mixture to the desired pH.

To justify the use of this method of inactivation of streptomycin as a step prior to sterility testing it must be shown that any organism resistant to 30,000 units of streptomycin per ml is also resistant to the concentration of semicarbazide present in the inactivation mixture. This question has been approached in 2 ways: Thus far we have found no vegetative cells resistant to such concentrations of streptomycin but have compared the sensitivities of a number of species to this antibiotic with their sensitivities to several carbonyl reagents at much lower concentrations of both substances. The results of such studies with 5 species are given in Table I. The second approach to this problem is based on the finding that spores of some species of bacteria are extremely re-

TABLE II. Recovery of *Bacillus sp.* No. 290 from Streptomycin Inactivated by Semicarbazide.

Tube No.	Mixture incubated at 25°C, 24hr					Mixture diluted in thioglycolate broth, incubated at 37°C, 48 hr. Growth after dilution in broth					
	Spore suspension* ml	Streptomycin† ml	Semicarbazide-hydrochloride‡ ml	Potassium acetate§ ml	H_2O	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	0.3	0.3	0.3 (undil.)	0.3	—	—	+	+	+	+	—
2	0.3	0.3	0.3 (½)	0.3	—	—	+	+	+	+	—
3	0.3	0.3	0.3 (¼)	0.3	—	—	+	+	+	+	—
4	—	0.3	0.3 (undil.)	0.3	0.3	—	—	—	+	+	—
5	0.3	—	0.3 "	0.3	0.3	—	—	—	+	+	—
6	0.3	0.3	—	—	0.6	—	—	—	—	—	—
7	0.3	—	—	0.3	0.6	—	+	+	+	—	—
8	—	0.3	—	—	0.6	—	—	—	—	—	—
9	—	—	0.3 "	—	0.9	—	—	—	—	—	—
10	—	—	—	0.3	0.9	—	+	+	+	—	—
11	0.3	—	—	—	0.9	—	—	—	—	—	—
12	—	—	—	—	1.2	—	—	—	—	—	—

* Spore suspension contained ca. 1,000,000 spores per ml.
† Streptomycin solution = 98,700 units per ml = 0.0287 g per ml.
‡ Semicarbazide-hydrochloride solution = 0.0862 g per ml in undiluted solution.
§ 5% potassium acetate solution.
+ = growth; — = no growth.

sistant to the germicidal action of streptomycin even though the growth of their vegetative forms is inhibited by very low concentrations of streptomycin in broth.[1] The spores of such an organism (*Bacillus sp.* No. 290) were added to a solution containing 28,700 units of streptomycin per ml and the streptomycin solution then was inactivated in the manner described. The results of a typical experiment are tabulated in Table II.

It will be seen in Table I that the growth-inhibiting concentration of semicarbazide ranges from 533 to >11,000 times greater than the inhibiting concentrations of streptomycin for the organisms tested.

Ability of spores of Bacillus sp. No. 290 to resist the action of semicarbazide during the inactivation of streptomycin. Various mixtures of spores, streptomycin, semicarbazide-hydrochloride and potassium acetate were prepared as shown in Table II and incubated at 25°C for 24 hours. Each mixture was then diluted by 10-fold steps in freshly prepared thioglycolate broth, the broth dilutions were incubated at 37°C, and read for growth after 24, 48 and 96 hours incubation. Little change occurred after 48 hours incubation; therefore only the 48-hour readings are recorded in the table.

It is shown in Table II that the streptomycin-spore suspension mixture gave no growth until diluted 10^{-4} in thioglycolate broth, whereas growth occurred at 10^{-2} in mixtures containing 3, 2 and 1 γ of semicarbazide hydrochloride per γ of streptomycin. When spores were added to semicarbazide-hydrochloride and diluted in thioglycolate broth, growth again occurred in the 10^{-2} dilution. Hence under the conditions of the experiment presented, streptomycin, containing viable cells, when treated with the specified carbonyl reagent yielded growth at 1/100th the dilution required to obtain growth from the original streptomycin-spore suspension mixture.

Summary. 1. Details are given for a method of inactivating streptomycin with semicarbazide-hydrochloride in order to test the sterility of concentrated streptomycin solutions. It has been shown that although several carbonyl reagents inhibit bacterial growth, it requires from 533 to >11,000 times more semicarbazide-hydrochloride (one of the least toxic of the group) to cause this inhibition than is required of streptomycin.

2. Spores of *Bacillus sp.* No. 290, when added to a streptomycin solution containing 28,700 units per ml, were able to grow out when diluted 10^{-4} in thioglycolate broth. When a similar spore suspension-streptomycin mixture was treated with semicarbazide-hydrochloride (3, 2 or 1 γ carbonyl reagent per unit (or γ) of streptomycin) and then diluted in thioglycolate broth, growth occurred at a 10^{-2} dilution.

15361

Buffers in the Range of pH 6.5 to 9.6.*

GEORGE GOMORI.
From the Department of Medicine, The University of Chicago.

In the pH range between 6.5 and 9.6, the buffers generally used have been phosphate, barbital,[1] ammonium salts and carbonate.[2] Among these, phosphate and carbonate are incompatible with Ca salts; ammonium salt buffers are not entirely stable; barbital, on account of its low solubility, can be prepared in low concentrations only and, in addition, inhibits certain enzyme systems.[3] Mertz and Owen[4] have suggested the use of imidazole as a buffer in the physiologic pH range, com-

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research at the University of Chicago.

1 Michaelis, L., *J. Biol. Chem.*, 1930, **87**, 33.

2 Delory, G. E., and King, E. J., *Bioch. J.*, 1945, **39**, 245.

3 Quastel, J. H., and Wheatley, A. H. M., *Proc. Roy. Soc. B.*, 1932, **112**, 60; *Bioch. J.*, 1934, **28**, 1251.

Method. Materials required for inactivation of streptomycin solution containing 30,000 units per ml ± 10%:

1. Semicarbazide-hydrochloride solution: ca. 0.06 g per ml. Sterilization through U. F. fritted glass filter is the preferred method. The sterile solution is stored at 4°C.

2. Potassium acetate solution: ca. 0.05 g per ml. Solution is sterilized by autoclaving, 10 lb pressure for 10 minutes.

Procedure for inactivation of streptomycin. To each ml of streptomycin solution to be inactivated is added 1 ml of the potassium acetate solution and then 1 ml of semicarbazide-hydrochloride solution, observing aseptic precautions. The mixture is incubated at ca. 25°C (room temperature) for 24 hours.

After room temperature incubation, the inactivation mixture is tested for sterility in freshly prepared thioglycolate broth, using at least 30 ml of broth for each ml of inactivation mixture to be tested. The inoculated broth is incubated at 37°C for at least 24 hours.

Discussion. It has been shown that 2 γ of semicarbazide hydrochloride per γ (or unit) of streptomycin will cause 98% inactivation at pH 5.3.[4] Since the semicarbazide-hydrochloride solution is extremely low in pH, potassium acetate is first added to the streptomycin solution to bring the final mixture to the desired pH.

To justify the use of this method of inactivation of streptomycin as a step prior to sterility testing it must be shown that any organism resistant to 30,000 units of streptomycin per ml is also resistant to the concentration of semicarbazide present in the inactivation mixture. This question has been approached in 2 ways: Thus far we have found no vegetative cells resistant to such concentrations of streptomycin but have compared the sensitivities of a number of species to this antibiotic with their sensitivities to several carbonyl reagents at much lower concentrations of both substances. The results of such studies with 5 species are given in Table I. The second approach to this problem is based on the finding that spores of some species of bacteria are extremely re-

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1	0.3	0.3	0.3 (undil.)	0.3		|	+++	+++	+++	+++	|
2	0.3	0.3	0.3 (½)	0.3		|	+	+	++	|	|
3	0.3	0.3	0.3 (¼)	0.3		|	+	|	|	|	|
4	—	0.3	0.3 (undil.)	0.3	0.3	|	|	|	|	|	|
5	0.3	—	0.3 "	0.3	0.3	|	|	|	|	|	|
6	0.3	0.3	— —	—	0.6	|	+	|	|	|	|
7	0.3	—		0.3	0.6	+	+	|	|	|	|
8	—	0.3		—	0.9	|	|	|	|	|	|
9	—	—	0.3 "	—	0.9	|	|	|	|	|	|
10	—	—	— —	0.3	0.9	|	|	|	|	|	|
11	0.3	—	— —	—	0.9	|	+	|	|	|	|
12	—	—	— —	—	1.2	+	|	|	|	|	|

* Spore suspension contained ca. 1,000,000 spores per ml.
† Streptomycin solution = 28,700 units per ml = 0.0287 g per ml.
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§ 5% potassium acetate solution.
+ = growth; — = no growth.

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		Mar.	3		
		Apr.	4		
	47	May	1		
				2	June
	48	Oct.	1		
				3	Dec.
1942	49			1	Jan.
				2	Feb.
				3	Mar.
				4	Apr.
	50			1	May
	51	Oct.	1		
		Nov.	2		
		Dec.	3		
1943	52			1	Jan.
		Feb.	2		
		Mar.	3		
		Apr.	4		
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TABLE I.
pH Values of Buffers at 23°C and 37°C.

0.1 N HCl cc	Collidine		Tris(hydroxymethyl)-aminomethane		2-amino-2-methyl-1,3-propanediol	
	23°C	37°C	23°C	37°C	23°C	37°C
*5.0	8.35	8.28	9.10	8.95	9.72	9.62
7.5	8.18	8.10	8.92	8.78	9.56	9.45
10.0	8.00	7.94	8.74	8.60	9.38	9.27
12.5	7.88	7.80	8.62	8.48	9.26	9.15
*15.0	7.77	7.70	8.50	8.37	9.15	9.03
17.5	7.67	7.60	8.40	8.27	9.05	8.94
20.0	7.57	7.50	8.32	8.18	8.96	8.85
22.5	7.50	7.40	8.23	8.10	8.87	8.76
*25.0	7.40	7.32	8.14	8.00	8.78	8.67
27.5	7.30	7.23	8.05	7.90	8.70	8.58
30.0	7.22	7.14	7.96	7.82	8.60	8.50
*32.5	7.13	7.05	7.87	7.73	8.50	8.40
35.0	7.03	6.95	7.77	7.63	8.40	8.30
*37.5	6.92	6.84	7.66	7.52	8.30	8.20
40.0	6.80	6.72	7.54	7.40	8.18	8.07
42.5	6.62	6.54	7.36	7.22	8.00	7.90
*45.0	6.45	6.37	7.20	7.05	7.83	7.72

patible with Ca; however, its high cost is almost prohibitive.

Three new buffers: 2,4,6-collidine, tris(hydroxymethyl)-aminomethane and 2-amino-2-methyl-1,3-propanediol, are suggested for the use in the pH range between 6.5 and 9.6. They are quite soluble, do not precipitate Ca salts, and are low in price. They were found to be stable at room temperature for a period of over 3 months. Collidine and Tris(hydroxymethyl)-aminomethane, to be used in the pH ranges between 6.5 and 8.3, and between 7.2 and 9.0, respectively, were tested by Dr. E. S. Guzmán Barrón for their effect on the O_2 uptake of rat kidney slices in the presence of 0.01 M pyruvate. The concentration of the buffers was 0.02 M. phosphate buffer being used as a control. The results with the different buffers were all well within the limits of experimental error, thus showing complete lack of inhibitory action. Tris(hydroxymethyl) - aminomethane and 2-amino-2-methyl-1,3-propanediol (range, pH 8.0 to 9.7) were tested for their effect on alkaline phosphatase at pH 9.1, 0.005 M glycerophosphate being used as a substrate. Barbital and Delory and King's[2] carbonate buffers served as controls. Again, no inhibitory effect was noted.

The pK_b values of the new buffer substances were determined by the electrometric determination of the pH of their half-neutralized 0.05 M solutions at 23°C and 37°C. The apparatus used was a Leeds and Northrup potentiometer with glass and calomel electrodes. Phthalate buffer served as a standard.

1. 2,4,6-collidine (s-collidine).† Colorless liquid; pH 7.4 at 23°C; 7.32 at 37°C, pK_b 6.6 and 6.68, respectively.

2. Tris(hydroxymethyl) - aminomethane.‡ Colorless crystals; pH 8.14 at 23°C; 8.00 at 37°C; pK_b 5.76 and 6.0, respectively.

3. 2-amino-2-methyl-1,3-propanediol.‡ Colorless, somewhat hygroscopic crystals; pH 8.78 at 23°C; 8.67 at 37°C; pK_b 5.22 and 5.33, respectively.

The pH values of 0.05 M buffer mixtures, obtained by mixing 25 cc of a 0.2 M solution of the bases (collidine, 2.64 cc in 100 cc; tris(hydroxymethyl)-aminomethane, 2.43 g in 100 cc; 2-amino-2-methyl-1,3-propanediol, 2.1 g in 100 cc) with varying volumes of 0.1 N HCl and diluting the mixtures to the final volume of 100 cc, are given in Table I. The values marked with an asterisk were determined by potentiometric measurement, all the other ones were interpolated by calculation. The effect of salts on these values was not determined.

[2] Mertz, E. T., and Owen, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 204.

† Obtainable from the Eastman Kodak Co., Rochester, N. Y.

‡ Obtainable from the Commercial Solvents Corporation, 17 East 42nd Street, New York.

15362 P

Therapeutic Effectiveness of Single Oral Doses of Penicillin.

H. J. WHITE, M. E. LEE, AND C. ALVERSON. (Introduced by Raymond N. Bieter).

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Very few chemotherapeutic agents are effective when administered as single oral doses in acute bacterial infections. Robinson, Smith and Graessle[1] reported 80% survival with a batch of crude streptomycin, using a single oral dose of about 1500 mg per kilo, in an acute *Salmonella* infection in mice.* These investigators also found that a single oral dose of about 500 mg per kilo of crude streptothricin (assumed potency 300 units per mg) saved 76% of mice in a similar infection.[2] Finally, each of several analogues of pantothenic acid, administered as a single oral dose of 500 mg per kilo, has produced 100% survival in an acute hemolytic streptococcal infection in mice.

It is the purpose of the present communication to report preliminary results of a study of the therapeutic effectiveness of single oral doses of penicillin.

Methods. The penicillin powder used in this work was labelled "Crude, Penicillin G, pH 5.0 salt." It was supplied by Lederle Laboratories, Pearl River, N. Y. Its potency was determined as 650 *Staphylococcus* units per mg. Thus, on the basis of 1667 units per mg of pure penicillin G, the purity of the powder was considered to be approximately 39%.

The untreated experimental streptococcal infection is characterized by rapidly developing septicemia uniformly terminating in death within 48 hours. Treated animals alive on the 21st day after infection are considered to be completely protected.

Results. The therapeutic effect which can be obtained with a single oral dose of penicillin is shown in Table I. It is evident that a single dose of only 25 mg (equivalent to 16,000 to 17,000 units) per kilo of mouse produced a marked effect (97% survival).

TABLE I.
Therapeutic Effectiveness of Single Oral Doses of Penicillin.

Organism: *Hemolytic streptococcus;* Group A; strain C203.
Mice: Vanderwerken; 18-22 g.
Infection: Intraperitoneal; 0.5 cc of a 10^{-5} broth dilution of a 6-hour TSP-blood broth culture; 4000 ± 400 organisms.
Treatment: Penicillin powder containing 650 units per mg; single dose by stomach tube immediately after infection.

Single dose mg/kg	Survival on 21st day after infection alive/total No.	Dose calculated as pure penicillin G mg/kg
4000	10/10	1600
2000	10/10	800
1000	10/10	400
500	10/10	200
250	30/30	100
200	30/30	80
150	30/30	60
100	30/30	40
50	30/30	20
25	29/30	10
Untreated controls	0/40	—

Discussion. The fact that 97% survival was obtained with crude penicillin powder (650 units per mg) administered orally as a single dose of 25 mg per kilo suggests clinical applications for streptococcal infections. Thus, an equivalent dose for a 60-kilo patient would be 1.5 g; expressed as pure penicillin G, this dose would be only 0.6 g.

[1] Robinson, H. J., Smith, D. G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 226.

* The dosage reported was 3000 units. The dose given above was calculated on the assumptions that the streptomycin sample contained 100 units per mg and that the average mouse weight was 20 g.

[2] Robinson, H. J., and Smith, D. G., *J. Pharm. and Exp. Therap.*, 1944, **81**, 390.

TABLE I.

Tissue	Stage	Estrus	Diestrus	Pseudopreg.			Pregnancy				Lactation					
				4	7	11	4	7	11	15	20	4	7	11	15	20	22	23
Avg wt. (mg) Corpora lutea		1.2	1.1	1.5	1.6	—	1.5	1.4	2.1	4.0	4.5	2.4† 1.5 15.9† ±.13	1.6 1.8 16.6 ±.76	0.7 1.5 24.9 ±.95	0.6 1.7 20.6 ±.43	0.5 2.1 16.5 ±.87	0.4 2.2 16.4 ±.88	0.5 2.9 15.4 ±1.3
Avg ATP-ase Units‡ Corpora Lutea		7.8 ±.47§	9.8 ±.60	7.2 ±.32	7.9 ±.54	8.1 ±.40	7.4 ±.15	7.8 ±.31	7.5 ±.17	5.8 ±.81	9.2 ±.17	5.5 ±.24	7.5 ±.65	7.8 ±.71	6.7 ±.28	9.1 ±.47	12.3 ±.57	9.8 ±.40
Avg ATP-ase Units‡ Ovarian residue		13.6 ±.54	15.7 ±.27	18.0 ±.25	17.6 ±.84	11.2 ±.57	13.5 ±.74	19.7 ±.66	13.8 ±.22	14.3 ±.82	12.6 ±.20	16.3 ±.64	17.7 ±.43	19.8 ±1.3	16.8 ±.59	15.0 ±.23	16.0 ±.78	14.7 ±.83

* Days. † First line, corpora of pregnancy which persist during lactation; second line, corpora lutea of the post-partum ovulation.
‡ A unit is the quantity of ATP-ase which liberates 1 γ of inorganic phosphorus from ATP in 15 minutes at 37°C. The values are expressed as units per mg of fresh tissue, each value being the average of 9 determinations (3 for each of 3 rats). § Standard error of the mean.

11th day of lactation, during which time the average weight of the corpora lutea decreased abruptly.

During the first 20 days of lactation the ATP-ase activity of the corpora lutea of the post-partum ovulation paralleled closely the activity of the corpora of pregnancy during gestation. The increased ATP-ase after day 20 was correlated with decreasing lactation and reappearance of estrous cycles.

When the above results are calculated on the basis of change per corpus luteum rather than change per mg of tissue, the weight and the ATP-ase activity increase at practically identical rates during the periods of gestation in which there is active progesterone secretion.[3,4] The same relationship holds for the corpora of the post-partum ovulation during the first 20 days of lactation. Immediately before parturition and late in lactation the relationship changes so that the rate of change in ATP-ase activity exceeds the weight change rate. Following parturition both the weight and ATP-ase activity decrease, but the latter lags considerably behind the former. These results indicate a parallel increase in size and ATP-ase activity of the functional lutein cell, which suggests, together with other evidence linking the ATP system with lipid metabolism,[5,6] that ATP-ase may be involved in the production of progesterone by the functional corpus luteum.

The enzyme activity of the ovarian residue during pregnancy increased to a high value on day 7, then decreased significantly on day 11, and remained approximately unchanged until after parturition. Following littering a maximum was reached on day 11 of lactation.

We have also studied the succinic dehydrogenase activity of lutein and ovarian tissues throughout pregnancy and lactation[7] to

[3] Atkinson, W. B., and Hooker, C. W., *Anat. Rec.*, 1945, **93**, 75.

[4] Laqueur, G. L., and Koets, P., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 239.

[5] Lardy, H. A., Hansen, R. G., and Phillips, P. H., *Arch. Biochem.*, 1945, **6**, 41.

[6] Lehninger, A. L., *J. Biol. Chem.*, 1945, **157**, 363.

[7] Unpublished data.

Such doses appear to be "massive" only when expressed as "units." The advantages of therapeutic administration of penicillin in a single oral dose, or in oral doses given once or twice a day, are obvious.

Further work is in progress to determine the effectiveness of single oral doses of different lots of penicillin in the streptococcal and other infections in mice.

Summary. Survival of 97% was obtained in a streptococcal infection in mice with crude penicillin powder containing 650 units per mg, administered in a single oral dose of 25 mg per kilo.

15363 P

Adenosine Triphosphatase Activity and Weight of Corpora Lutea During Reproductive Cycle of the Rat.*

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Adenosine triphosphate (ATP) and the ATP-splitting enzyme, adenosine triphosphatase (ATP-ase), have been found in tissues other than muscle,[1] and evidence has been presented that the ATP system links the energy-requiring reactions of tissues with the energy-yielding reactions.[2] These results suggested an investigation of the ATP-ase activity of ovarian structures since these tissues undergo rapid changes in growth and function during which large amounts of energy must necessarily be involved.

Materials and Methods. The method of DuBois and Potter[1] was used in determining the ATP-ase activity of corpora lutea and remaining ovarian tissue of female rats during estrus, diestrus, pseudopregnancy and lactation at the intervals indicated in Table I. This method measures the amount of inorganic phosphorus released by the enzyme of homogenized tissue when ATP is used as substrate in a buffered solution (pH 7.4) containing calcium. The corpora lutea were dissected from the ovaries using sharp-pointed scalpel blades and spectacle loupes. The identification of the corpora of pregnancy and corpora of the post-partum ovulation throughout lactation was based on the differences in size, shape and color. That these criteria were adequate was confirmed by marking the corpora of pregnancy with fine silk thread. The corpora and ovarian residue were weighed and homogenized prior to adding to the incubation mixture. Each ATP-ase value in the table is the average of 9 determinations (3 for each of 3 rats), and each weight is the average of the corpora lutea obtained from 3 rats.

Results and Discussion. In diestrus there was a slight increase in the ATP-ase of the corpora lutea and ovarian residue above the estrus level. The average weight of the diestrous corpora was essentially the same as that found in estrus. During pseudopregnancy the weight of the corpora increased somewhat from the estrus level, but the ATP-ase activity did not change significantly. As pseudopregnancy progressed a definite decrease in the value of the ovarian residue was noted. At 11 days estrus was imminent, and the results suggest an inverse relationship between estrogen level and ATP-ase of the extra-luteal tissues.

The ATP-ase activity of the corpora lutea during pregnancy, when calculated on a per mg of tissue basis, did not show marked variation despite the fact that the average weight of the corpora lutea progressively increased. Late in pregnancy an increase in activity was found which continued until the

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

2 Potter, V. R., *J. Cell. and Comp. Physiol.*, 1945, **26**, 87.

TABLE I.
Total Daily Urinary 17-Ketosteroids.

	Control period, normal sleep days			Experimental period, no sleep days			
	1st	2nd	3rd	1st	2nd	3rd	4th
Group A							
Med.	—	—	—	Plac.	Plac.	Plac.	Plac.
KA	20.8	23.6	24.9	22.6	20.9	21.5	18.5
EB	17.0	18.1	15.7	14.0	16.1	14.8	17.0
BB	11.8	13.1	11.9	12.7	11.5	15.2	11.5
LC	21.1	22.7	23.4	18.6	18.4	19.0	21.2
DM	14.2	17.1	14.5	17.2	19.1	17.6	14.6
RT	19.1	19.3	19.3	20.6	16.7	10.7	13.7
Mean	(17.4)	(19.0)	(18.2)	(17.6)	(17.1)	(16.5)	(16.1)
Group B							
Med.	—	—	—	Amytal	Amytal	Plac.	Plac.
BA	17.6	21.8	19.8	18.8	14.4	17.3	17.9
MD	15.4	14.7	13.5	13.0	10.1	11.4	13.3
CE	20.7	20.7	23.7	22.9	17.8	20.6	17.9
EF	16.0	15.4	19.7	17.1	14.3	16.4	16.5
DL	10.5	12.9	12.3	14.6	12.0	9.2	12.3
TL	17.1	21.0	18.7	16.3	17.5	19.3	18.0
Mean	(16.1)	(17.8)	(18.0)	(17.1)	(14.4)	(15.7)	(16.0)

Values expressed as mg dehydro-iso-androsterone.

after. The other group (A) received placebos throughout. All received the same food and participated in the same activities.[1]

Urine was collected, first as a control under normal conditions of sleep, for 3 days prior to the experiment, and during the sleepless period of 112 hours, as follows: Each man saved his entire output for each 24-hour period in a bottle containing 10 cc of concentrated HCl. Each sample was acidified immediately after the termination of each 24-hour period with a volume of concentrated HCl equivalent to 15% of the total urine volume, and stored at 2-4°C.

Pincus' procedures for hydrolysis and extraction,[3] and the colorimetric method of Callow[4] were employed. The concentration of total 17-ketosteroids was calculated by means of a calibration curve which had been established with graded amounts of dehydro-iso-androsterone acetate. Suggestions by Talbot *et al.*[5,6] and Engstrom and Mason[7] were followed for correction of the results for the influence of non-ketonic chromogens. In 13 representative samples, the non-ketonic fraction was separated from the ketones by the use of Girard's reagent T: from the colorimeter readings obtained with these samples, a calibration curve was drawn for the correction of all other samples.

Results and Discussion. The results are given in the table, and indicate that:

Prolonged wakefulness did not change the level of the urinary 17-ketosteroids. The values for each individual of the placebo group, as well as for this group as a whole, were not significantly different for the control and the experimental periods. This is in agreement with a previous, but incomplete, study made by one of us. (D.B.T.) on 20 subjects in which prolonged wakefulness

[1] In order to keep the men awake for prolonged periods, it is necessary that they be on a program of continuous physical activity. This is particularly important after the first day. This experiment is one of many dealing with prolonged wakefulness involving almost 700 subjects. Because of the interest at the moment in 17-ketosteroid output during various conditions of stress, it seems to be desirable to report the results of this phase of the study at the present time.

[3] Pincus, G., and Pearlman, W. H., *Endocrinol.*, 1941, **29**, 413.

[4] Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

[5] Talbot, N. B., Butler, A. M., MacLachlan, E. A., and Jones, R. N., *J. Biol. Chem.*, 1940, **136**, 365.

[6] Talbot, N. B., Berman, R. A., and MacLachlan, E. A., *J. Biol. Chem.*, 1942, **143**, 211.

[7] Engstrom, W. W., and Mason, H. L., *Endocrinol.*, 1943, **33**, 229.

extend previous studies of this enzyme system in these tissues.[8] In these studies it was also found that the dehydrogenase activity varies with the stage of the reproductive cycle, there being a steady increase in the QO_2 of the corpora lutea of pregnancy until midterm after which this level is maintained until parturition. During lactation the value declines rapidly to the early pregnancy level and remains there throughout 20 days of suckling. The QO_2 of the corpora lutea of the post-partum ovulation increases until day 20 of lactation when the highest value obtained from any ovarian tissue is reached. The QO_2 of the ovarian residue does not vary markedly during pregnancy or lactation.

Summary. The ATP-ase activity and weight of lutein and ovarian tissue were determined during estrus, diestrus, pseudopregnancy, pregnancy and lactation. The results indicate that in general the enzyme activity per unit weight is lower in functional corpora lutea than in apparently non-functional corpora. The weight and enzyme activity per corpus luteum increased and decreased at approximately the same rate during pregnancy and lactation. The enzyme activity of ovarian tissue remaining after the removal of the corpora lutea was greatest on the 4th day of pseudopregnancy, 7th day of pregnancy and 11th day of lactation.

[8] Meyer, Roland K., McShan, W. H., and Erway, Wilma F., *Endocrinology*, 1945, **37**, 431.

15364

Effect of Prolonged Wakefulness on the Urinary Excretion of 17-Ketosteroids.*

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Selye[1] has shown that a number of different types of stress cause a stimulation of the adrenal cortex. As a result of this stimulation, the adrenal secretes more substances of steroid nature, and the level of 17-ketosteroids in the urine is increased. Pincus and Hoagland[2] demonstrated such an increase in the output of 17-ketosteroids in pilots under conditions of stress produced by actual flight.†

Prolonged wakefulness may be considered a condition which causes considerable stress on the organism. If the response of the adrenal cortex to stress were a general phenomenon, it would be expected that prolonged wakefulness would induce a similar stimulation of the cortex with a resulting increase in the excretion of 17-ketosteroids.

Methods. Twelve subjects,‡ ranging in age from 18 to 33 years, were used. They were divided into 2 groups of 6 each. One group (B) received 1 grain of amytal§ every 12 hours for the first 48 hours of the sleepless period and then placebos every 12 hours there-

* The work described in this report was done under contract recommended by the CMR, between the Office of Scientific Research and Development and the California Institute of Technology.

[1] Selye, H., *Endocrinol.*, 1937, **21**, 169.

[2] Pincus, G., and Hoagland, H., *J. Aviat. Med.*, 1943, **14**, 173.

† After this MS. was completed, Pincus *et al.*[3] reported that the 17-ketosteroid excretion is reduced during sleep.

‡ Twelve conscientious objectors of the Glendora Civilian Public Service Camp volunteered for this and other experiments carried out under this project, and their cooperation was excellent.

§ Amytal was given in this experiment to determine its effect on the performance of men who might be forced to remain awake for prolonged periods of time. It was a constituent of a motion sickness preventive being considered for general use by the armed services.

TABLE II.
Treatment of Pneumococcus Type III Pneumonia in Mice.

Date	Time	Six mice used in each group					
		Controls	Treated immediately	Treated after 1 hr	Treated after 3 hr	Treated after 6 hr	Treated after 9 hr
2/19/46	9 a.m.	Infected	Infected	Infected	Infected	Infected	Infected
"	9 "	—	0.5 cc subtilin	—	—	—	—
"	10 "	—	—	0.5 cc subtilin	—	—	—
"	12 m.	—	0.5 cc "	"	0.5 cc subtilin	—	—
"	3 p.m.	—	"	"	"	0.5 cc subtilin	—
"	6 "	—	"	"	"	"	0.5 cc subtilin
"	9 "	—	"	"	"	"	"
2/20	9 a.m.	1st dead	"	"	"	"	"
"	12 m.	2nd and 3rd dead	"	"	"	"	"
"	3 p.m.	—	"	"	"	"	"
"	6 "	4th dead	"	"	"	"	"
"	9 "	5th and 6th dead	"	"	"	"	"
2/21	5 a.m.	—	"	"	"	"	"
3/7	Results	All dead	All living	All living	All living	All living	All living

tion was shown to be bacteriostatic in high dilution and bactericidal in more concentrated solution.

In the present communication subtilin was tested for its effect upon Type III pneumococcus *in vivo*.

Experimental. Nine white mice, weighing between 20 and 25 g, were injected intraperitoneally with 0.1 cc of a 24-hour serum broth culture of *Diplococcus pneumoniae*, Type III. Three of the mice were not treated but served as controls; 3 were treated immediately; the remaining 3 were treated one hour later. Each mouse was injected intraperitoneally with 0.5 cc of a solution containing 0.1 mg subtilin per cc (½ unit)[2] and every 4 hours thereafter throughout the first day. Three injections were given on the second day and one on the third. The schedule of treatments and results obtained are recorded in Table I. It may be seen that all of the control mice died in about 24 hours. On the other hand, all of the treated animals were living 2 weeks after treatment was discontinued. The animals were discarded after this period of time.

In a second series a larger number of animals (36) was used to check the results obtained in the first experiment. Each mouse was injected intraperitoneally with 0.1 cc of a 24-hour serum broth culture of Type III pneumococcus. The treated animals were each given 0.5 cc of a solution containing 0.1 mg subtilin per cc. Six mice were not treated but used as controls; 6 were treated immediately; 6 were treated after an interval of 1 hour; 6 were treated after 3 hours; 6 after 6 hours; and 6 after 9 hours. The animals were given another treatment 3 hours after the first injection, and every 3 hours thereafter until 9 p.m., then continued the following morning. The treatments were continued for approximately 48 hours. The results are recorded in Table II. It may be seen that all of the control mice died in from 24 to 36 hours after being given an injection of Type III pneumococcus. On the other hand all of the treated mice survived after being given subtilin for only 48 hours.

[2] Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 23.

appeared to have no significant effect on the urinary 17-ketosteroids.

The administration of 1 grain of amytal every 12 hours during the first 48 hours of the sleepless period resulted in a slight but noticeable depression in the excretion of these substances. This was most noticeable on the 2nd day. Upon stopping the amytal administration there was a gradual rise to the pre-amytal or control values.

Total urine volumes varied greatly between individuals as well as from day to day. The amount of ketosteroid excreted appeared to be practically independent of the total urine volume.

The observations that fatigue from sleeplessness did not influence the urinary excretion of 17-ketosteroids are in contradiction to the findings of Pincus and Hoagland,[2] and Pincus, Hadidian and Yeaton.[8] It must be pointed out, however, that in our experiments the output of an entire 24-hour period was measured, while the results of Pincus and Hoagland were based on samples collected during relatively short periods of stress. Furthermore, the type of stress was different and, therefore, the results are not strictly comparable.

Summary. 1. The urinary excretion of 17-ketosteroids of normal young men was not influenced by prolonged wakefulness (112 hours). 2. Amytal given during such an experiment slightly depressed the output of these substances.

[8] Pincus, G., Hadidian, Z., and Yeaton, M., *Federation Proc.*, 1946, **5**, 81.

15365

Subtilin—Antibiotic Produced by *Bacillus subtilis*.* III. Effect on Type III Pneumococcus in Mice.†

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In a previous communication[1] subtilin was found to exert a pronounced antibiotic action against a number of types of the pneumococcus by the agar cup-plate procedure. This ac-

TABLE I.

Treatment of Pneumococcus Type III Pneumonia in Mice.

		3 mice used in each group		
Date	Time	Controls	Treated immediately	Treated after 1 hr
2/14/46	12:30 p.m.	Infected	Infected	Infected
,,	12:30 ,,	—	0.5 cc subtilin	—
,,	1:30 ,,	—	,,	0.5 cc subtilin
,,	5:30 ,,	—	,,	,,
,,	9:30 ,,	—	,,	,,
2/15	8:00 a.m.	First dead	—	—
,,	9:30 ,,	—	0.5 cc ,,	0.5 cc ,,
,,	12:30 pm	Second ,,	,,	,,
,,	4:00 ,,	Third ,,	—	—
,,	5:30 ,,	—	0.5 cc ,,	0.5 cc ,,
2/16	9:30 a.m.		0.5 cc ,,	0.5 cc ,,
3/2	Results	All mice dead	All mice living, apparently normal	All mice living, apparently normal

* This investigation was aided by a grant from Eli Lilly and Company, Indianapolis, Indiana.

† The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, Calif.

[1] Salle, A. J., and Jann, Gregory J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 60.

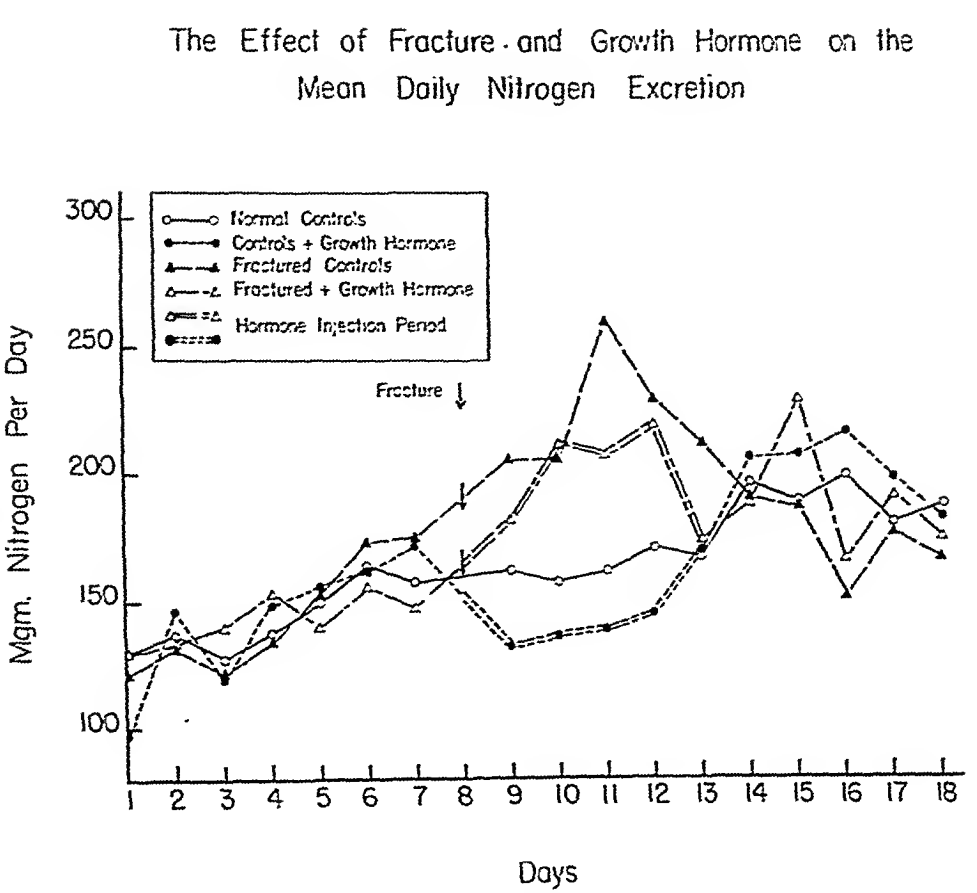

Fig. 1.

scribed.[6]

Following a preliminary period of adaptation to diet and cages, the nitrogen excretion was followed daily for a 7-day control period. At this time, both femurs were fractured under ether anesthesia. An incision was made on the lateral aspect of the thigh: the muscles were freed from the bone: and a comminuted fracture of approximately ½ cm in length was produced in the middle third of each femur. The incision was then sutured. There was little bleeding, and, although rigid asepsis was not observed, there was no evidence of infection. Daily intraperitoneal injections of 1 mg of growth hormone were begun on the day of fracture and were continued for 5 days. The daily nitrogen excretion was followed for 5 more days after growth hormone injections were stopped. Nitrogen was determined by the micro-Kjeldahl method.

Results. In Fig. 1 are shown the curves of mean daily nitrogen excretion for the 4 groups. The normal control group showed a slight but steady rise in nitrogen excretion over the entire period. In the control period before fracture, the lines representing the nitrogen excretion of the 4 groups intermingled, but at the 8th day they separated characteristically. That of the unoperated group given growth hormone dropped abruptly after the first injection and remained low during the injection period, but rose above that of the normal controls after growth hormone was stopped. In the fractured group, the excretion rose on the first day after fracture, reached a maximum on the third, and fell off in the following few days. In the last

[6] Li, Choh Hao, Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, 150, 353.

The 6 mice in the last group (9-hour interval) were in very bad condition before treatment was started. After the second or third injection they appeared almost normal and after 24 hours they appeared to be free from any symptoms of pneumonia. All of the mice were observed for an additional 14 days without any treatment, then discarded. The antibiotic did not produce any observable toxic reaction in the mice.

Conclusions. Subtilin has been shown to exert a powerful *in vivo* action on the course of experimental pneumococcus Type III infections in mice. Animals treated with subtilin 9 hours after being injected with the organism were quickly cured of the infection. The antibiotic exhibited no apparent toxic reaction in the animals.

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Effect of Anterior Pituitary Growth Hormone on Urinary Nitrogen Loss Following Fracture.*

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Introduction. It was noted by Cuthbertson[1] that bone fracture in man produced a rise in urinary nitrogen excretion, which, at its height, was not affected by a high protein diet. It was shown that this was due to more than disuse atrophy, as splinting of the limbs in healthy individuals produced only a slight rise. Howard and co-workers[2] have confirmed Cuthbertson's work in man, and have demonstrated that there was a long period (average 35.6 days) during which patients were in actual negative nitrogen balance. In a subsequent publication[3] these same workers found no beneficial effect from increased protein in the diet, the added protein being excreted quantitatively in the urine.

In rats, Cuthbertson[4] noticed a similar rise following fracture. The maximum nitrogen excretion occurred on the third day, and the animals had returned to normal after 5 days. Cuthbertson also found that the amount of nitrogen excreted was not accounted for by the loss of weight in the limb, but rather that tissue breakdown seemed to be occurring generally throughout the body. Cuthbertson, Webster and Young[5] found that a crude alkaline extract of the anterior pituitary prevented the rise in nitrogen excretion. In view of these findings it seemed of interest to determine whether any effect could be obtained with growth hormone, inasmuch as it would be expected to cause nitrogen retention.

Methods. Rats of the Long-Evans strain approximately 50 days old were used. In order to ensure a constant food intake during the whole experimental period, all animals were restricted to 10 g of stock diet per day. Twenty-four males were studied and were divided into 4 groups of 6 each. The first group was retained as a normal control, the second unoperated but given growth hormone, the third operated, and the fourth operated and given growth hormone. The growth hormone was a homogeneous preparation and was isolated by the procedure previously de-

* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation, New York City.

1 Cuthbertson, D. P., *Lancet*, 1942, **1**, 433.

2 Howard, J. E., Parson, W., Stein, K. E., Eisenberg, H., and Reidt, V., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 156.

3 Howard, J. E., Winternitz, J., Parson, W., Bigham, R. S., Jr., and Eisenberg, H., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 209.

4 Cuthbertson, D. P., McGirr, J. L., and Robertson, J. S. M., *Quart. J. Exp. Physiol.*, 1939, **29**, 13.

5 Cuthbertson, D. P., Webster, T. A., and Young, F. G., *J. Endocrin.*, 1941, **2**, 468.

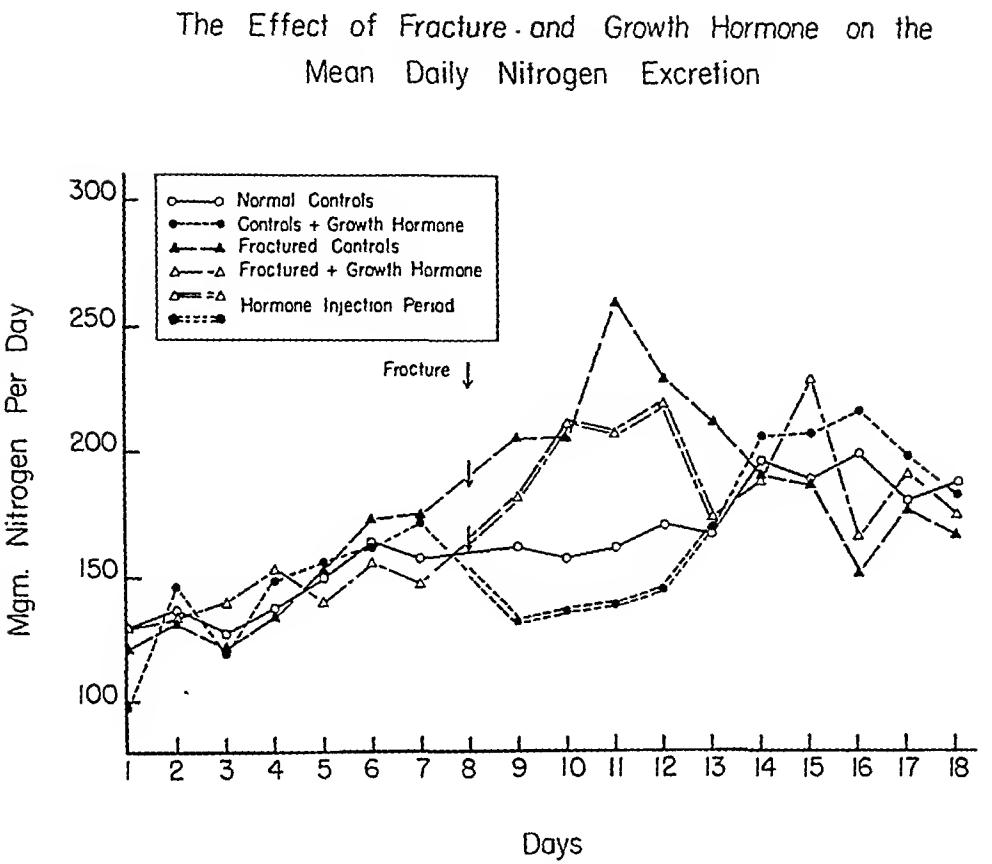

Fig. 1.

scribed.[6]

Following a preliminary period of adaptation to diet and cages, the nitrogen excretion was followed daily for a 7-day control period. At this time, both femurs were fractured under ether anesthesia. An incision was made on the lateral aspect of the thigh; the muscles were freed from the bone; and a comminuted fracture of approximately ½ cm in length was produced in the middle third of each femur. The incision was then sutured. There was little bleeding, and, although rigid asepsis was not observed, there was no evidence of infection. Daily intraperitoneal injections of 1 mg of growth hormone were begun on the day of fracture and were continued for 5 days. The daily nitrogen excretion was followed for 5 more days after growth hormone injections were stopped. Nitrogen was determined by the micro-Kjeldahl method.

Results. In Fig. 1 are shown the curves of mean daily nitrogen excretion for the 4 groups. The normal control group showed a slight but steady rise in nitrogen excretion over the entire period. In the control period before fracture, the lines representing the nitrogen excretion of the 4 groups intermingled, but at the 8th day they separated characteristically. That of the unoperated group given growth hormone dropped abruptly after the first injection and remained low during the injection period, but rose above that of the normal controls after growth hormone was stopped. In the fractured group, the excretion rose on the first day after fracture, reached a maximum on the third, and fell off in the following few days. In the last

[6] Li, Choh Hao, Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1945, **159**, 353.

group, fractured and growth hormone treated, the rise occurred but was less than that in the fractured untreated group.

The mean level of nitrogen excretion for each of the 4 groups during the control period was essentially the same, running from 142 mg to 144 mg of nitrogen per day with a standard deviation of from ± 3.5 to ± 5.1. In the 5-day postoperative period the average daily excretion of the control group rose to 165 ± 4.8† while that of the control group given hormone rose only to 145 ± 4.3. This represents a daily retention of 20 mg of nitrogen for the treated group as compared with the controls for the same period. The average daily excretion of the fractured group rose from 142 ± 5.1 to 223 ± 9.0, while that of the group fractured and given growth hormone rose only from 143 ± 3.5 to 199 ± 8.0, representing a retention over the untreated fractured group of 24 mg of nitrogen per day. The differences between the groups noted in this first 5-day postoperative period were all significant having p values of .05 and less.[7]

In the 5-day period after growth hormone was stopped, the nitrogen excretion of the control group averaged 30 mg per day higher than in the previous period, while that of the growth hormone treated group rose 58 mg. Also, the nitrogen excretion of the group fractured and given growth hormone was higher on the average than that of the fractured untreated group.

Discussion. In these experiments the retention of nitrogen following growth hormone was approximately the same in the fractured and unfractured rats when they were compared with their respective untreated controls. It would appear that the effect of growth hormone was to lower the base line of nitrogen excretion upon which the stress of fracture was superimposed. Cuthbertson, Webster and Young[5] reported that a crude extract of the anterior pituitary completely prevented the rise of nitrogen excretion after fracture, although it did not promote nitrogen storage beyond that occurring in the uninjected control period. There are several important differences between our experiments and theirs which could explain the different results obtained. In the present experiment 2 legs were fractured, creating a greater tendency to breakdown of protein than in the experiment of Cuthbertson, Webster and Young, where only one leg was broken. In addition younger animals were used in our experiments, which may have reduced the nitrogen retaining effect of the growth hormone.

Summary. The daily urinary nitrogen excretion was followed in growth hormone treated rats with bilateral femur fractures. The normal and fractured rats given growth hormone showed approximately the same decrease in nitrogen excretion when compared with their respective controls.

† Standard deviation of the mean.

[7] Fisher, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, London, 1936.

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Measured Dose of Gamma Hexachlorocyclohexane (γ 666) Required to Kill Flies and Cockroaches, and a Comparison with DDT.

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Hexachlorocyclohexane† or "666" has been said to be more toxic than DDT‡ for certain insects[2,5,7,8,9,11] and mammals.[3] As was also true of DDT until recently,[13] however, most of the information is in terms of percent kill after exposure for a given time to a given environmental concentration of the

* This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

agent. There are very few data available[4] on the actual measured dose (mg per kg) required to kill insects. Exposure data are invaluable from the point of view of field application,[6] but for studies on mechanism of action, it is essential to know the approximate dose which an insect must receive to produce death or some other specific physiological effect.[14,15] Therefore, measurements have been made of the LD_{50} (mg per kg required to kill 50%, determined graphically and by the method of Bliss[1]) of the active gamma isomer of hexachlorocyclohexane for flies *(Musca domestica* and *Calliphora spp.)* and the cockroach *(Periplaneta americana)*.

Methods. Since the gamma isomer of hexachlorocyclohexane is far more toxic than the alpha, beta or delta forms, and is the important constituent of manufactured 666, it has been used in pure form (m.p. 112.5°C)[12] throughout, in these studies. All roaches have been unanesthetized, but flies were lightly anesthetized with ether during the administration of the toxic agent.

Methods of administration have been essentially the same as those used in a similar study of DDT.[13] The toxic agent, dissolved in acetone, was delivered through a 27-gauge hypodermic needle, from a 0.25 cc tuberculin syringe fitted with a micrometer screw-driven piston.[10] Such an instrument is capable of delivering volumes of liquid of the order of 0.2 mm^3 with about 5% accuracy. For injection purposes, in the roach, the needle was made to enter the abdomen beneath a sternite and near its hinge-like point of attachment. Upon withdrawal of a needle so inserted the sternite falls back into place and seals the hole. No leakage has been seen following such a procedure. For surface application, roaches were held by the wings. The γ 666 was then deposited beneath the wings on the dorsum of the thorax from the instrument described above, but now through a blunted needle held flat against the body surface. When folded back into place the seldom-moved wings protect against loss of dry powder remaining after solvent evaporation. Control animals were given acetone without the γ 666 (Tables I and II).

Roaches were kept in large battery jars, in groups of 10 or fewer, with food and water continuously available. Flies were kept in similar groups in 150 cc wide-mouthed bottles.

Results. The detailed data are shown in Tables I and II, and are summarized and compared with similar data for DDT in Table III. It will be seen (Tables I and II) that in the region of the LD_{50}, changes in dose of γ 666 made relatively little difference in terms of mortality. This plateau-like region of the toxicity curve is thought to be due to gross inhomogeneity of the test animals. Neither sexes nor specific ages were carefully selected and random variations in

† 1, 2, 3, 4, 5, 6, hexachlorocyclohexane or "666" is a mixture of alpha, beta, gamma and delta isomers. The name "Gammexane" refers to the active gamma isomer as does "γ 666."

‡ 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane.

[1] Bliss, C. I., *Ann. Appl. Biol.*, 1935, **22**, 134.

[2] Bracey, P., and David, W. A. L., (British) *Insecticide Development Panel*, 1944, (44), 170.

[3] Cameron, G. R., and Burgess, F., (British) *Insecticide Development Panel*, 1944, (44), 131.

[4] David, W. A. L., (British) *Insecticide Development Panel*, 1945, (45), 236.

[5] Gersdorff, W. A., and McGovran, E. R., *Soap and Sanitary Chemicals*, 1945, **21**, 117.

[6] Jenkins, D. W., U. S. Army Service Forces, Chemical Warfare Service, Medical Division Report No. 56. Edgewood Arsenal, Md., Sept. 26, 1945.

[7] Kearns, C. W., Ingle, L., and Metcalf, R. L., *J. Econ. Ent.*, 1945, **38**, 661.

[8] Madden, A. H., Lindquist, A. W., and Jones, H. A., *U. S. Dept. of Agric. Bur. Ent. and Plant Quar. Int. Rep.*, No. 0-101.

[9] McGovran, E. R., Gersdorff, W. A., Fales, J. H., and Piquett, P. G., (British) *Insecticide Development Panel*, 1944, (44), 209.

[10] McMaster, P. D., Rockefeller Inst. for Med. Research, N. Y. C., personal communication.

[11] Richards, A. G., and Cutkomp, L. K., *Biol. Bull.*, in press.

[12] Slade, R., (British) *The Hurter Memorial Lecture, Insecticide Development Panel*, 1945, (45), 237.

[13] Tobias, J. M., Kollros, J. J., and Savit, J., *J. Pharm. and Exp. Ther.*, 1946, **86**, 287.

[14] Tobias, J. M., and Kollros, J. J., in press.

[15] Tobias, J. M., Kollros, J. J., and Savit, J., in press.

TABLE I.
Toxicity of γ 666 for the Cockroach (*Periplaneta americana*).

Route	No. roaches	Vol. given mm³	γ 666 mg per kg	% mortality at hours indicated 24	48	72	96	120
Surface	10	1.0	0	0	0	0	0	0
	20	1.0	1	10	10	15	20	20
	22	0.4-1.0	4	36	45	50	50	50
	25	0.6-1.0	6	28	52	56	56	56
	10	0.8	8	60	70	80	80	80
	49	1.0	10	18	45	49	57	59
	20	1.0-2.0	20	20	60	65	70	75
Injection	10	1.0	0	0	0	0	0	0
	10	1.0	1	0	10	10	20	20
	10	1.0	4	20	50	60	70	70
	10	1.0	6	20	20	30	30	40
	20	1.0	10	20	65	75	80	80
	10	1.0	20	30	60	70	80	80

Graphically determined
Approximate 120-hr LD_{50} (surface), 5 mg per kg (range 4 to 7.5).
Approximate 120-hr LD_{50} (injection), 4 mg per kg (range 3 to 7.5).
By method of Bliss[1]
120-hr LD_{50} (surface), 4.57 mg per kg (fiducial limits 1.0-9.4 mg per kg).
120-hr LD_{50} (injection), 3.39 mg per kg (fiducial limits less than 0.5-1.3 mg per kg).

TABLE II.
Toxicity of γ 666 Applied to the Body Surface of Flies (in Acetone).

Species	No. flies	Vol. applied mm³	γ 666 mg per kg	% mortality at hours indicated 24	48
Musca domestica (newly emerged, 1-18 hr)	25	0.4	0.3	16	20
	25	0.6	0.4	52	60
	25	0.8	0.5	56	60
	22	1.0	0.7	91	95
(older adults)	31	1.0	0	3	3
	20	1.0	0.3	5	5
	30	0.4	0.5	30	30
	30	0.6	0.8	33	33
	50	0.6	1.4	64	67
	15	1.0	1.8	87	100
	10	0.4	2.7	90	90
	5	1.0	3.5	100	—
	20	0.6	6.0	100	—
	19	1.0	7.0	100	—
Calliphora spp. (older adults)	33	0.4	0.5	24	42
	20	0.6	0.7	10	60
	32	0.8	0.9	41	65
	15	1.0	1.2	80	100

Graphically determined
Approximate 48-hr LD_{50} (*Musca*-newly emerged), 0.4 mg per kg (range 0.4 to 0.5).
Approximate 48-hr LD_{50} (*Musca*-older adults), 1 mg per kg (range 0.7 to 1.2).
Approximate 48-hr LD_{50} (*Call. spp.*-older adults), 0.6 mg per kg (range 0.6 to 0.7).
By method of Bliss
48-hr LD_{50} (*Musca*-newly emerged), 0.4 mg per kg (fiducial limits less than 0.1-0.65 mg per kg).
48-hr LD_{50} (*Musca*-older adults), 0.83 mg per kg (fiducial limits, 0.63-1.1 mg per kg).
48-hr LD_{50} (*Call. spp.*-older adults), 0.6 mg per kg (fiducial limits, less than 0.1 to more than 1.2 mg per kg).

TABLE III.
Comparative Toxicity of γ 666 and DDT for Flies and Cockroaches.*

Insect	Approximate LD_{50}, mg per kg†			
	Surface		Injection	
	γ 666	DDT	γ 666	DDT
Periplaneta americana	5	10	4	5-8‡
Musca domestica				
(newly emerged)	0.4	2	—	—
(older adults)	1	8-21	—	—
Calliphora spp.				
(older adults)	0.6	9-28	—	—

* DDT data.[13]
† Roach observation period 120 hours, fly 48. Solvent acetone in all cases.
‡ Probably closer to 8.[13]
N.B. All data in this table graphically determined.

either or both of these categories could account for such a spread.

From Tables I and III it can be seen that the LD_{50} for γ 666 applied to the surface of the cockroach (4.6 mg per kg) is not significantly different from that for intra-abdominal injection (3.4 mg per kg). This approximate equivalence in toxicity of surface-applied and of injected material is a most interesting phenomenon, and has also been demonstrated, in the case of the cockroach, for DDT.[13] It is not known to the authors to occur with any toxic agent administered to mammals. The finding again highlights the importance of the extremely efficient absorption of certain substances by the insect body surface. It is most unlikely that this absorption took place through spiracles, since in the roach, where application was easily localizable, the toxic agent was placed on the body at a considerable distance from the spiracles, and inspection revealed rapid evaporation of solvent with little flow. In the case of DDT, in addition, there has been shown to be a positive correlation between the presence of a chitinous exoskeleton and susceptibility to the external application of the toxic agent.[11]

Gamma 666 is only about twice as toxic as DDT for the cockroach (Table III). For the adult fly, however, it is distinctly more toxic (LD_{50}, 0.8 mg per kg) than DDT (LD_{50}, 8 to 28 mg per kg). The data show that it is also somewhat more toxic ($p = 0.05$) for the newly emerged fly *(Musca domestica)* (LD_{50}, 0.4 mg per kg) than for the adult (LD_{50}, 0.8 mg per kg), but the decrease in sensitivity as the fly ages is less than that reported for DDT (Table III).[13] The toxicity data reported here suggest a somewhat greater absolute toxicity of γ 666 for adult flies than do those of David[4] who reported a maximum median lethal dose of 2 and 3 mg per kg for the male and female respectively of *Musca domestica*. The difference is small, however, and may not be a meaningful one.

Those flies *(Musca domestica)* and roaches *(Periplaneta americana)* which die after a surface applied LD_{50} dose of γ 666 do so more quickly than after a comparable dose of DDT.[13] In addition, although the times have not been measured, it is the strong impression of the authors that these insects show symptoms of poisoning (tremors, ataxia, convulsions, falling, prostration) much sooner after γ 666 than after a comparably toxic dose of DDT. This is important, since a faster "knockdown rate" implies less time for ranging and oviposition.

Conclusions. 1. The approximate LD_{50} for γ 666 in the cockroach *(Periplaneta americana)* is 4.6 mg per kg when applied to the body surface and 3.4 mg per kg when injected intra-abdominally. These values are not significantly different.

2. An approximate equivalence, similar to that noted above, between surface and injection toxicity for the roach has been reported for DDT. This finding emphasizes the importance of the absorptive capacity of the insect body surface for contact poisons

in contributing to the effectiveness of insecticides.

3. The approximate LD_{50} for γ 666 applied to the body surface is 0.4 mg per kg for the newly emerged fly *(Musca domestica)*. For the older adult the LD_{50} is about 0.8 mg per kg for *Musca domestica* and 0.6 mg per kg for *Calliphora spp.* This decrease in sensitivity as the fly ages is less than that reported for DDT.

4. Gamma 666 is about twice as toxic as DDT for the cockroach *(Periplaneta americana)*, and is distinctly more toxic than DDT for *Musca domestica* (newly emerged and adult) and *Calliphora spp.* (adult).

5. Both death and knockdown occur more rapidly after surface application of γ 666 to *Musca domestica* and *Periplaneta americana* than after DDT.

15368

Type-specific Capsular Swelling of Meningococci by Chicken Antiserum.

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Branham[1] has recently emphasized the value of serologic typing of meningococci for epidemiologic studies as well as for more exact etiologic diagnosis. The methods currently employed for this purpose include (a) capsular swelling with hyperimmune rabbit serum and (b) agglutination with monovalent antisera prepared in rabbits or chickens. One of the advantages of using chickens as a source of agglutinating serum is their ability to tolerate doses of meningococci which may be toxic or even lethal for rabbits.[2,3]

During the past 18 months we have had occasion to prepare such antisera in adult chickens. The course of injections was based on the report of Phair, Smith and Root.[2] Doses of 2 to 4 billion living organisms, derived from casein-hydrolysate starch agar cultures[4] and suspended in physiological saline, were given intravenously at intervals of approximately one week. After 4 injections the animals were allowed a rest period of 7 to 16 days before being bled; in some instances this bleeding was followed after 7 to 9 days by a fifth injection and the animals were kept 1 to 4 weeks longer before final exsanguination. The serum collected from the successive bleedings of each chicken was pooled and stored at 2°C. Throughout the period of immunization the birds appeared healthy and showed no appreciable loss of weight.

In testing for agglutinins, meningococci from cultures grown 5 to 18 hours on casein-hydrolysate starch agar were suspended in saline to a concentration of 2 to 4 billion cocci per ml. Dilutions of antiserum were mixed with an equal volume (0.3 ml) of culture suspension in small tubes and these were agitated vigorously on a Kahn shaking machine for 10 to 20 minutes at room temperature before readings were made. Agglutination was readily visible with the naked eye. The titer of the serum was recorded as the highest dilution, after addition of antigen, in which clumping was marked. The results summarized in Table I show that following immunization of chickens with 16- to 18-hour cultures or with cultures only 4 hours old, we obtained satisfactory agglutinating sera versus strains of meningococci

1 Branham, S. E., *Am. J. Pub. Health*, 1945, **35**, 232.

2 Phair, J. J., Smith, D. G., and Root, C. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 72.

3 Miller, C. P., *Yale J. Biol. and Med.*, 1944, **16**, 519.

4 Mueller, J. H., and Hinton, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 330.

TABLE I.
Summary of Data on Immunization of Chickens with Meningococci.

Animals employed			Immunizing antigens			Type-specific serologic properties of antiserum	
No.	Breed	Sex	Serologic type	Strain	Age of culture (hr)	Capsular swelling	Agglutination titer
68	Rhode Island Red	♀	I	1027	16-18	+	120
1	Plymouth Rock	♂	I	1027	4	+	240
7	White Rock	"	I	1027	4	+	120
66	Rhode Island Red	♀	II	963	16-18	0	80
49	" " "	"	II α	1054	16-18	+	40
3	Buff Orpington	♂	II α	1054	4	+	160
8	White Rock	"	II α	1054	4	+	320

Types I, II and II alpha, as others have done.[2,3,5]

Investigation was then made to determine whether these agglutinating sera would also exhibit the property of capsular swelling as is the case with rabbit anti-meningococcal sera of adequate potency. Suspensions of living organisms from 4- or 5-hour cultures on Mueller-Hinton medium were mixed in the usual fashion with a loopful of chicken antiserum plus a loopful of methylene blue stain. Microscopic observation revealed prompt and strikingly clear-cut capsular swelling of meningococci Types I and II alpha in the presence of their homologous antisera; no capsular swelling was demonstrable when cocci of these serologic types were mixed with any heterologous antiserum. No capsular swelling was seen in the mixtures of Type II meningococci with the single homologous serum available or with the several antisera versus the other 2 types. The above findings have been checked and found to hold true not only for the various sera as freshly drawn but also after storage at 2°C for periods from 8 to 14 months. The data in Table I also indicate that sex or breed does not markedly affect the success of immunization providing healthy adult birds, weighing 5 to 7 pounds, are employed.

Discussion. So far as we are aware, data on the use of chickens for the preparation of specific capsular swelling antisera against meningococci or other bacterial species have not been previously reported. The present findings are of interest in demonstrating that capsular swelling may be produced with antisera derived from animals other than the rabbit and the horse; they also suggest that chickens can be used to prepare quellung sera for bacteria to which the rabbit or horse may not respond favorably.

From a practical standpoint, the ease with which adult chickens can be successfully immunized for the production of quellung antiserum versus meningococci Types I and II alpha should make it feasible for many diagnostic laboratories to prepare a supply. Such sera can undoubtedly be used for rapid and direct serologic identification of meningococci in the spinal fluid as well as in cultures obtained from other sources.

[5] Kabat, E. A., Miller, C. P., Kaiser, H., and Foster, A. Z., *J. Exp. Med.*, 1945, **81**, 1.

15369

The Activity of Anionic Surface Active Compounds in Producing Vascular Obliteration.

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The great variety of substances used for obliteration of varicose veins have the common property of being destructive to intima of the vein.[1,2] Some of the substances are

used in a strongly hypertonic concentration (glucose, sodium chloride) and dehydrate through osmosis; others form a highly alkaline solution which is incompatible with cell life even for a relatively short period of time (sodium carbonate, soap solutions); still others contain substances which are poisonous to the protoplasma of cells in general (quinine hydrochloride, halogens, mercuric chloride). Differences in the manner in which the intima of the vein is destroyed are suggested by the differences in the rate of thrombus formation after injection. Mercuric chloride produces thrombus in 3 or more days,[3] whereas complete obliteration takes place within 24 hours after the injection of 20% sodium salicylate solutions.[4] Even more pronounced are the effects produced by soap solutions.[1] As this may be owing at least in part to the surface activity of the fatty acid anions and the anionic micelles formed in aqueous solutions of soaps, it was of interest to study the effectiveness of the synthetic anionic detergents in obliterating veins. The results of this study are reported in the following.

Methods. The marginal ear vein of rabbits and the veins of dogs have been used frequently to study the thrombogenic and sclerosing properties of various agents.[5,6,7]

In preliminary experiments using the marginal ear vein of rabbits it was found that it is difficult to regulate the rate of dilution of the sclerosing solution with blood, which obviously is one of the important factors determining the extent of thrombus formation. Because of the great number of collaterals, dilution of the agent after injection was rapid and high concentrations of the agent had to be used. These destroyed the delicate marginal ear vein and its collaterals rapidly and completely, permitting free diffusion of the agent and producing inflammatory reactions and necrosis of the surrounding tissue. Even if thrombus was produced without much tissue irritation, it was often extended to collaterals and its length could not easily be measured.

Much better results were obtained when the tail vein of the mouse was injected. Here, again, high concentrations of the agents, 2% of sodium ricinoleate or the equivalent in sclerosing strength of the anionic synthetic detergents, caused gangrene within 24 to 48 hours and the entire tail sloughed off within a few days. However, if the injection was carried out at a very slow rate, thrombus could be produced using lower concentrations of most of the agents without much tissue irritation.

In order to obtain well reproducible averages it was necessary to standardize the procedure as follows: mice weighing 20 to 25 g were fasted for 20 hours before injection. They were placed in a holder which permitted free handling of the tail. The veins were distended by dipping the tail for 1 minute into water which was kept at a temperature of 45°C. The solution was injected from a 0.25 cc Tuberculin Syringe No. LT ¼ with divisions of 0.01 cc through a 26-gauge needle having a short bevel. The needle was inserted at a distance of 50 mm from the base of the tail so that the tip of the needle was about 45 mm from the base of the tail. Five-hundredths cc was injected over a period of 10 seconds. Dilution of the solution with blood was prevented for 50 seconds by compressing the vein at the base of the tail and at the site of the injection. The length of the thrombus was measured with a ruler. In the dose range used, thrombus was usually formed within 24 hours. Delayed thrombus formation was observed occasionally with the lowest concentrations used. The optimum time for reading giving the maximum average length of thrombus was at about 48 to 96 hours, although the differences in results obtained when the reading was made at any time

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2 McPheeters, H. O., and Anderson, J., *Injection Treatment of Varicose Veins and Hemorrhoids*, F. A. Davis Company, Philadelphia, 1943.

3 Régard, G. L., *Rev. med. de la Suisse Rom., Genevé*, 1925, **65**, 102.

4 von Meisen, *Acta chir. Scandinav., Stockholm*, 1926, **60**, 435; 1927, **62**, 17.

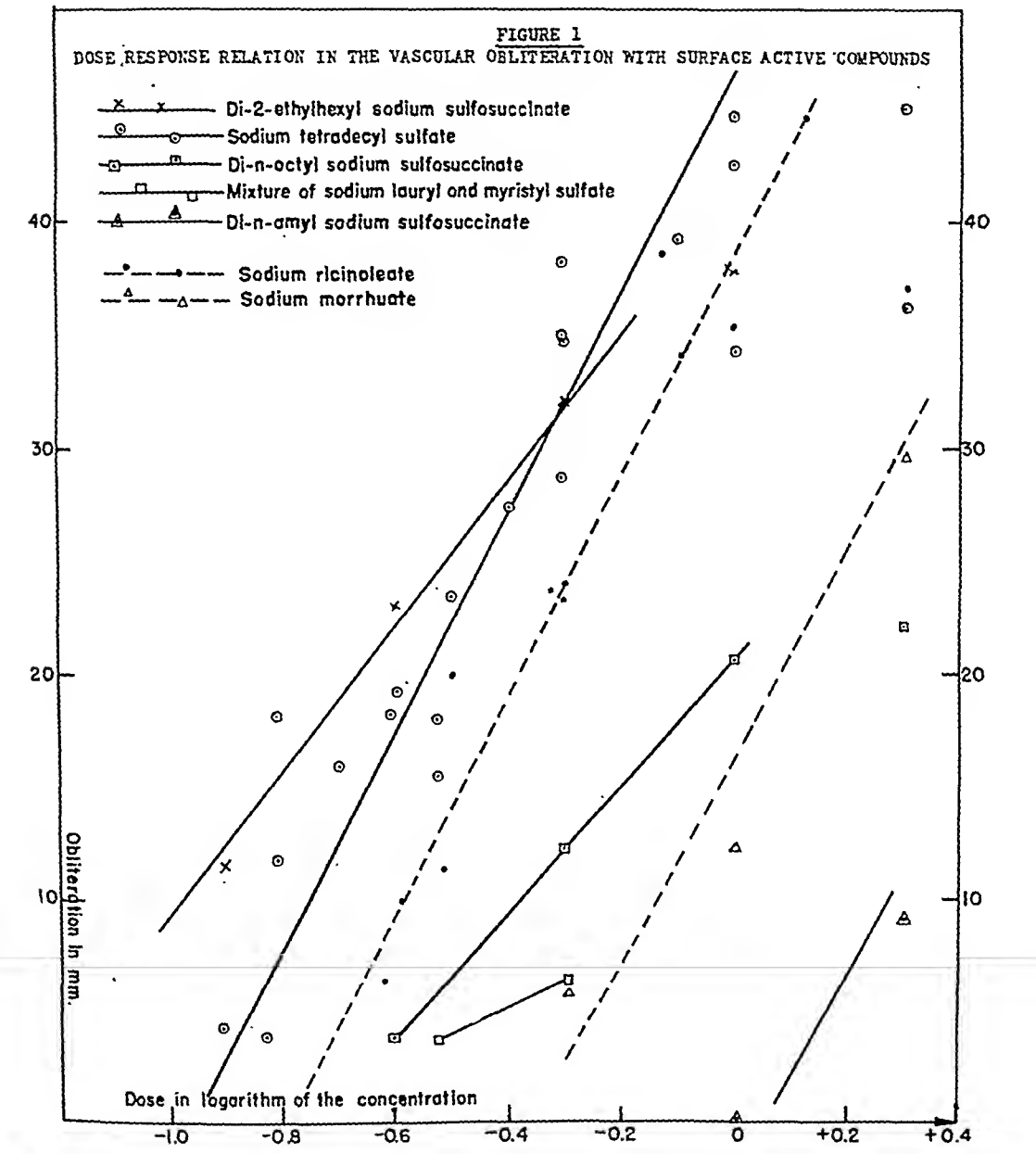
5 Kilbourne, N. J., Dodson, V., and Zeller, A. H., *Surg. Gynec. and Obst.*, 1932, **54**, 640.

6 Isaak, L., *Arch. Dermat. and Syphilol.*, 1940, **42**, 86.

7 Jensen, H., and Jamke, P., *J. Am. Pharm. A. (Scient. Ed.)*, 1944, **33**, 362.

FIGURE 1

DOSE RESPONSE RELATION IN THE VASCULAR OBLITERATION WITH SURFACE ACTIVE COMPOUNDS



between 2 to 7 days were not significant. The experimental points in Fig. 1 are averages of 2 to 3 readings made within 1 week after the injection. Each point represents the average length of the thrombus calculated from the results obtained with usually 10, but not less than 4 mice.

The average length of the thrombus decreased gradually during the latter part of the first week and subsequently. In the low-response range recanalization was frequent. In the high-response range permanent obliteration through organization took place. The extent of obliteration after resorption of the thrombus was measured in a few instances by dipping the tails in warm water, thereby

distending the patent veins, and it was found to be approximately proportional to the obliteration caused by thrombosis.

Materials Used. Extensive tests using 130 mice were made with a commercial sodium ricinoleate solution, pH 8.3, containing 5% of the soap and 2% of benzyl alcohol in water. Dilutions of 0.15 to 2.0% made with sterile distilled water were used.

The sodium morrhuate tested was also a commercial preparation containing 5% of the soap and 2% of benzyl alcohol in water. The solution was slightly turbid and had a pH of 10.1. Dilutions containing 2.0, 1.0, 0.5 and 0.25% of the soap were made with sterile distilled water. Ten mice were injected with each of these dilutions.

Sodium tetradecyl sulfate (sodium 2-methyl-7-ethyl-undecyl sulfate-4) was prepared by purification of the commercial product. It was a colorless, transparent, waxy solid which contained 90% of the detergent; the remaining 10% consisted of phosphate buffer and water. Water-clear solutions of pH 7.5 were made with water or 0.85% aqueous sodium chloride solution. They were sterilized by autoclaving for 20 minutes at 15 lb pressure. This procedure did not cause appreciable hydrolysis of the ester as the solutions remained water clear and their pH value remained the same or decreased only slightly. One hundred and seventy mice were used and the concentration of the solutions injected varied from 0.125 to 2%.

For testing the effectiveness of sodium dodecyl and sodium n-tetradecyl sulfate 0.5% and 0.3% solutions of Duponol were used. The pH of the solutions was 7.1. Ten animals were injected with each of the 2 solutions.

Four of the aerosol type of compounds, the di-2-ethylhexyl (aerosol OT) (I), di-n-octyl (II), diethylpropyl (III), and di-n-amyl (IV) sodium sulfosuccinates were obtained in a highly purified form from the American Cyanamid Company.* Clear, approximately neutral solutions in distilled water were prepared by warming, if necessary, and keeping at 40°C until injected. Ten mice were injected with each of the solutions containing 0.125, 0.25, 0.50 and 1.0% of I; 0.25, 0.50, 1.0 and 2.0% of II; and 1.0 and 2.0% of III and IV.

Results. In Fig. 1 the average length of thrombus is plotted against the logarithm of the concentration. It seems that a correlation exists between the concentration and the response, *i.e.*, the average length of the thrombus. With sodium tetradecyl sulfate and the soap solutions, the response was proportional to the logarithm of the concentration, *i.e.*, dose, up to a response of about 40 mm. The maximal individual response was about 45 mm as this was the length of the vein exposed to the drug. The slope of the rectilinear portion of the dose response curve was about the same for these substances. From the estimated position of the lines, the ratio of potencies, that is, the reciprocal of the ratio of the doses producing equal responses, was estimated to be 1.5 for sodium tetradecyl sulfate over sodium ricinoleate. The preparation of sodium morrhuate used in this study seemed to be considerably weaker in thrombogenic activity than either sodium tetradecyl sulfate or sodium ricinoleate.

The slope of the straight portion of the curve obtained with the 2 isomeric aerosols, I and II, appears to be smaller than the slopes obtained with the soap solutions or sodium tetradecyl sulfate solution, although the difference may not be significant. The branched isomer, I, was found to be a much stronger thrombogenic agent than the normal chain isomer, II. In low concentrations I was more active than sodium tetradecyl sulfate. The dipentyl sodium sulfosuccinates, III and IV, were much less active than the dioctyl compounds. Here the branched chain compound, IV, showed no activity in the concentration used, whereas the normal chain compound, III, showed a slight activity. The mixture of the normal chain sodium dodecyl and tetradecyl sulfate was much less active than the branched chain sodium tetradecyl sulfate.

Toxicity to Tissues. This was tested by injecting aqueous solutions of the compounds in concentrations given in Tables I and II in doses of 0.1 cc subcutaneously and in doses of 0.05 cc intradermally into rabbits. The

* Through the courtesy of Mr. Clyde Sluhan.

TABLE I.

Reactions Produced by the Subcutaneous Injection of Soap Solutions and Sodium Tetradecyl Sulfate. (Figures: diameters in mm; dose: 0.1 cc; reading: 48 hours after injection).

Concentration, %	Edema			Erythema		
	Sor	STS	Morrh	Sor	STS	Morrh
5	15	0	8	15	0	8
2.5	0	0	11	0	0	11
1.25	0	0	8	0	0	8
0.625	0	0	0	0	0	0

Sor: Sodium ricinoleate.
STS: Sodium tetradecyl sulfate.
Morrh: Sodium morrhuate.

TABLE II.

Reactions Produced by the Intradermal Injection of Soap Solutions and Sodium Tetradecyl Sulfate. (Figures: diameters in mm; dose: 0.05 cc; reading: 48 hours after injection).

Concentration %	Necrosis			Hemorrhage			Edema			Erythema		
	Sor	STS	Morrh	Sor	STS	Morrh	Sor	STS	Morrh	Sor	STS	Morrh
5	11	5	8	6	3	7	13	6	10	13	6	15
2.5	10	7	6	0	2	0	14	12	13	14	12	14
1.25	9	3	6	0	0	0	11	3	12	12	8	12
0.625	6	0	6	0	0	0	9	6	6	9	6	11
0.313	4	0	6	0	0	0	6	0	6	6	0	11

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results in Table I show that sodium tetradecyl sulfate produced no appreciable irritation upon subcutaneous injection and that it was definitely less irritating than the soap solutions. The intradermal injections did not show significant differences among these compounds although the edema and erythema produced by sodium tetradecyl sulfate appeared to be less than that produced by soaps.

Acute Toxicity. Purified sodium tetradecyl sulfate (Tergitol-4) was found by Smyth *et al.*,[8] to have a toxicity of $LD_{50} = 2$ gm/kg when tested on rats by feeding a 25% aqueous solution by stomach tube. In testing the toxicity of Aerosol OT in mice Lorenz *et al.*,[9] found that 1 mg but not 2.5 mg is tolerated when given intraperitoneally and that 1.25 mg was lethal to 1 out of 25 mice weighing 25-30 g. The oral LD_{50} of Aerosol OT in mice has been reported to be 1.5 g/kg.[10]

The intravenous route of administration was chosen for acute toxicity determinations as this is used for therapy with these substances. The mice were fasted for 24 hours and injections of 0.1 cc of various dilutions were given over a period of 20 seconds. Most deaths occurred within 24 hours and no change of survival rate was found after 1 week. LD_{50} was estimated graphically,[11] to be 90 ± 5 mg/kg for sodium tetradecyl sulfate using 47 mice, 100 ± 30 mg/kg for Aerosol OT using 15 mice, 100 ± 3 mg/kg for sodium ricinoleate using 24 mice, and 150 ± 45 mg/kg for sodium morrhuate using 35 mice. Thus only slight differences were found in the toxicity of these substances. The value obtained for Aerosol OT is in fair agreement with the results of Lorenz *et al.*,[9] ($LD_{50} > 40$ and < 100 mg/kg), in view of the fact that the rate of injection which was not specified by these authors might have been greater than that used in these experiments.

Chronic Toxicity. Smyth *et al.*,[8] reported that sodium tetradecyl sulfate did not pro-

[8] Smyth, H. F., Seaton, J., and Fischer, L., *J. Indust. Hygiene and Toxicol.*, 1941, **23**, 478.

[9] Lorenz, E., Shimkin, M. B., and Stewart, H. L., *J. National Cancer Institute*, 1940, **1**, 353.

[10] Benaglia, A. E., Robinson, E. J., Utley, E., and Cleverdon, M. A., *J. Indust. Hygiene and Toxicol.*, 1943, **25**, 175.

[11] Miller, L. D., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 261.

distending the patent veins, and it was found to be approximately proportional to the obliteration caused by thrombosis.

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mal movements of men in training and have also established themselves among Army Air Force personnel.[4] Strains of only 3 Lancefield types, in order of degree of sulfonamide resistance, 17, 19 and 3, have been isolated under the circumstances just described.[3,4] Similar properties have been discovered in a few strains of other types, including 6 and 14.

The development of epidemics of respiratory infection caused by sulfonamide resistant hemolytic streptococci has been explained by 2 quite different hypotheses. One suggests that the etiological agents possessed a natural ability to multiply in the presence of sulfonamides and were therefore able to initiate outbreaks of disease in a host population in which sulfonamide prophylaxis had been established. Alternatively, it is proposed that mutants of previously sensitive strains appeared during rapid epidemic transmission which were resistant to the effect of sulfonamides. Such mutations were enabled to survive and spread because their new property was advantageous in the chemically altered host environment.

It seemed possible that a study of the sulfonamide resistance of a large number of strains of hemolytic streptococci collected during a study carried out between December 12, 1943 and April 20, 1944 in 2 army posts might give information as to which of the above concepts is correct. There was little possibility that sulfonamide resistant streptococci could have developed in naval activities and been transferred in so short a time to these particular army installations, since both were situated in an area remote from any naval training station. The nature and personnel of Post I has been described elsewhere.[5] Post II was an Army Air Base located about 10 miles from Post I. Contact between the 2 was possible through the intermingling of troops in a neighboring community.

Methods. Hemolytic streptococci were isolated from the upper air passages of infected men by technics described elsewhere.[6] The organisms were divided into groups and types by the precipitin technics of Lancefield. Sulfonamide sensitivity was determined by the method of Wilson;[7] penicillin sensitivity by streaking the streptococci on segments of blood agar plates containing appropriate amounts of the drug.

Results. Sulfonamide Sensitivity. The Group A hemolytic streptococci available for study consisted of 77 single, and 271 paired cultures of the same type, isolated from the upper air passages of 348 infected men at Post I. Each pair was recovered from the same individual, separate isolations having been made in the various cases at intervals from 1 to 25 days. In addition, 41 strains obtained from as many cases at Post II were tested.

Identical values for sulfonamide sensitivity were obtained with 225, and one-tube differences with 46, pairs. There were two-tube differences in 4 and these pairs were excluded. There was found no relationship between the time of isolation of the culture or the therapeutic administration of a sulfonamide and the variations between members of the pairs. It seems proper to assume that each pair of cultures represents the same organism recovered on 2 different occasions. The results indicate that the method for determination of sulfonamide sensitivity is quite precise and that relatively small differences in resistance may be satisfactorily compared.

The results of the determination of sulfonamide sensitivity of the single strains and of the nonidentical pairs (considering each member of the pair as a single strain) are presented in the first 4 columns of Table I. The values obtained for the 225 identical pairs are recorded in the 5th to 8th columns. The whole is summarized in columns 9 to 13.

No strain grew in the presence of more than 5 mg of sulfadiazine. Those of 5 types, 19, 24, 26, 30, and 36, as well as those included with the miscellaneous types, occasionally were

[4] a. Mitchell, R. B., Van Ravenswaay, A. I., Special Report, Office of the Air Surgeon, Oct. 8, 1945; b. Connor, A. R., Special Report, Office of the Air Surgeon, Oct. 8, 1945.

[5] Rantz, L. A., Rantz, H. H., Boisvert, P. J., and Spink, W. W., *Arch. Int. Med.*, in press.

[6] Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Arch. Int. Med.*, in press.

[7] Wilson, A. T., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 130.

duce appreciable toxicity when approximately 150 mg/kg were taken daily in the drinking water by rats for 30 days. Benaglia *et al.*,[10] gave doses varying from 0.19 to 0.87 g/kg to rats with the food and doses between 0.1 and 0.5 g/kg to rabbits, monkeys, and dogs for 24 hours without finding appreciable toxicity, although some inhibitory effect on the growth rate was discernible.

Repeated intravenous injection with these solutions could not be carried out. Among the practicable routes in mice, intraperitoneal injection most closely approximates the intravenous route. Hence, 0.1 mg sodium tetradecyl sulfate in 0.1 cc was injected into 19 mice first intravenously, then 12 times intraperitoneally in the course of 3 weeks. Four mice died of peritonitis or intercurrent infection during the first week. The average weight of the remaining mice increased from 25.9 g to 31.3 g. Five of the surviving mice showed localized infections (abscess in the spleen, localized peritonitis, and adhesions). These were obviously due to perforation at the time of injection. Sections made of the lungs, liver, kidney, spleen, brain, intestine, stomach, testis, and heart of the remaining 10 mice showed no pathology attributable to the drug.†

Summary. The thrombogenic activity of some soaps and synthetic detergents was compared by injecting their solutions under standardized conditions into the tail vein of mice. Sodium tetradecyl sulfate (2-methyl-7-ethyl-undecyl sulfate-4) and Aerosol OT (di-2-ethylhexyl sodium sulfosuccinate) were found to be more potent agents than the soap solutions generally used to sclerose veins. Alkyl sulfates and sulfonates containing large branched chain hydrophobic residues were found to produce thrombus more readily than their straight chain isomers. Sodium tetradecyl sulfate produced less tissue reaction than sodium ricinoleate or sodium morrhuate. Its toxicity was not significantly greater than that of the soap solutions studied.

† Thanks are due Dr. I. E. Gerber, pathologist, for the preparation and examination of the slides.

15370

Sulfonamide and Penicillin Resistance of Group A Hemolytic Streptococci.*

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It has frequently been possible to establish satisfactory prophylaxis against Group A hemolytic streptococcus respiratory infection and its complications by the daily administration of a sulfonamide.[1] Such a technic, established by the U. S. Navy in many activities early in December, 1943, was uniformly satisfactory[2] until mid-summer of 1944. At that time strains of hemolytic streptococci that were resistant to the antibacterial action of these agents appeared[3] as the cause of disease among personnel in a station in the northwest and initiated an epidemic in spite of the widespread use of chemoprophylaxis. Later these organisms were transmitted widely to other Navy activities through the nor-

* This study was carried out under the auspices of the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 U. S. Department of Labor, Children's Bureau Publication No. 308, Washington, 1945, 64-87.

2 NAVMED 284, Bureau of Medicine and Surgery, Navy Department, Washington, D. C., 1944.

3 a. Epidemiology Unit No. 22, *J. A. M. A.*, 1945, **129**, 921; b. Damrosch, D. S., *J. A. M. A.*, 1946, **130**, 124; c. Eekles, L. E., personal communication, Sept. 28, 1945.

must, therefore, be regarded as having been naturally resistant to the action of these chemicals.

Strains of 2 of these types, 3 and 17, later became established as the etiological agents of epidemic hemolytic streptococcal respiratory infection in military establishments in which sulfadiazine was being administered prophylactically. When studied under these circumstances the degree of resistance of strains of type 3 was approximately that discovered in this study but that of strains of type 17 was much greater.[3,4]

It is probable, therefore, that both of the hypotheses previously mentioned, explaining the appearance of epidemic, sulfonamide resistant, hemolytic streptococci, are correct. Certain strains of Group A hemolytic streptococci were present in the national military population at the time that mass chemoprophylaxis was instituted that possessed two advantageous biological properties: moderate sulfonamide resistance and a high degree of communicability. These characteristics permitted these strains to spread and cause disease after their introduction into a host population undergoing sulfonamide prophylaxis. In some instances (type 17) mutation occurred and much more sulfonamide resistant variants appeared; in others (type 3) the resistance of the strain did not become greatly enhanced.

This study has not revealed a precursor for the resistant strains of type 19 that also caused epidemics. It is quite possible, however, that naturally resistant variants of this type were present in other areas.

The failure of other moderately resistant strains discovered during this study to establish epidemics may have been due to a lack of certain properties which permit a high degree of communicability or to the fact that they were never introduced into a group in which sulfonamide prophylaxis was in use.

No significantly penicillin-resistant strains of hemolytic streptococci were discovered during this study.

Summary. Strains of certain types of Group A hemolytic streptococci were discovered to be naturally resistant to moderate amounts of sulfadiazine. It is suggested that such organisms originated the epidemics of streptococcal disease among troops receiving sulfonamide prophylaxis, later becoming more resistant by mutation. No strains significantly resistant to penicillin were discovered.

15371

Effect of Atropine, Testosterone and Pitressin on Experimental Myocardial Infarction.*

S. S. MINTZ AND B. KONDO.† (Introduced by L. N. Katz).

From the Cardiovascular Department, Research Institute, Michael Reese Hospital, Chicago.

Recently Mokotoff and Katz[1] have made use of an experimental approach to determine the effect of drugs upon infarct size following coronary ligation. It was shown by this means that papaverine had a definite effect in reducing infarct size, while aminophyllin parenterally had a smaller, but definite effect. It was felt that other drugs might profitably be evaluated by this method. In the present report data obtained with atropine, testosterone, and pitressin are presented.

Atropine was chosen since it has been claimed recently to have a clinically beneficial effect in myocardial infarction.[2] This beneficial effect was attributed to an abolition of supposed vagus coronary constrictor tone.

* Aided by the A. D. Nast Fund for Cardiovascular Research. The department is supported in part by the Michael Reese Research Foundation.

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[1] Mokotoff, R., and Katz, L. N., *Am. Heart J.*, 1945, **30**, 215.

[2] Leroy, G. V., Fenn, G. K., and Gilbert, N. C., *Am. Heart J.*, 1942, **23**, 637.

TABLE I.

Ability of Strains of Group A Hemolytic Streptococci to Grow in the Presence of Sulfadiazine.

Type	Single strains and individual members of nonidentical pairs considered as single strains				Identical pairs (2 cultures of same typo from same patient)				All strains (considering identical pairs as single strains)				
	No. of strains	Grew in control only	Grew in 1 mg	Grew in 5 mg	No. of pairs	Grew in control only	Grew in 1 mg	Grew in 5 mg	Total strains	Grew in 1 but not in 5 mg	Grew in 5 mg	% grew in 1 mg or more	% grew in 5 mg
1	14	3	11	0	12	3	7	2	26	18	2	76.9	7.5
3	23	7	9	7	17	7	6	4	40	15	11	65.0	27.5
6	9	2	5	2	3	0	3	0	12	8	2	66.6	16.7
17	16	1	5	10	20	0	3	17	36	8	27	97.5	75.1
19	21	12	9	0	38	30	8	0	59	17	0	28.8	0.0
24	2	2	0	0	8	7	1	0	10	1	0	10.0	0.0
26	5	3	2	0	15	15	0	0	20	2	0	10.0	0.0
30	10	6	4	0	19	19	0	0	29	4	0	13.8	0.0
36	24	13	9	2	33	33	0	0	57	9	2	19.3	3.5
44	5	4	1	0	6	3	1	2	11	3	2	36.4	18.2
46	8	0	4	4	12	2	7	3	20	11	7	90.0	35.0
Other types	32	17	13	2	42	30	11	1	74	24	3	36.5	4.0

resistant to the action of 1 mg per 100 ml of sulfadiazine but all but 5 strains failed to multiply in 5 mg per 100 ml of the drug. Strains of types 1, 3, 44, and 46 were often resistant to the action of 1 mg per 100 ml and, frequently, to that of 5 mg per 100 ml of sulfadiazine. Type 6 is represented by very few examples but nearly all were slightly sulfonamide resistant. The strains of type 17 were the least sensitive in that all but one grew in the presence of 1 mg per 100 ml and 27 of 36 in the presence of 5 mg of sulfadiazine per 100 ml.

Five strains of type 3 were collected at Post II; 4 grew in the control tube only, one in the 5 mg tube. Seventeen strains of type 19 were studied, only one of which grew in the 1 mg tube and none in the higher levels of sulfonamide. Seventeen of 19 strains of type 17 grew in the 1 mg and 4 in the 5 mg tubes.

The month of isolation of the resistant strains of types 3 and 17 was determined. All strains of type 3, able to grow in the presence of 5 mg of sulfadiazine, were isolated after March 1, 1944, since this type did not appear as a cause of disease in the post before this date. The results with type 17 were different in that 2 resistant strains were recovered in January and 6 in February of 1944.

Penicillin Sensitivity. The ability of all of the above listed strains to grow in the presence of penicillin was studied. Regarding them as individual strains, 54 were resistant to the action of 0.02, and 7 to that of 0.05 units per ml. All were inhibited by 0.1 unit per ml. If the paired strains only are considered, it is discovered that in only 5 of the 271 instances were both members of the pair able to grow in the presence of as much as 0.02 units of penicillin per ml. No relationship between the serological types of these slightly penicillin resistant strains was discovered.

Comment. This study has demonstrated that certain types of Group A hemolytic streptococci were significantly more resistant to the antibacterial action of sulfonamides than were others collected during the same period. It is unlikely that these strains had been transmitted through a host population undergoing sulfonamide prophylaxis. They

must, therefore, be regarded as having been naturally resistant to the action of these chemicals.

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Previous work from this department,[3] however, has shown that the vagi actually are tonic coronary dilators. Testosterone was chosen because clinical benefit had been claimed from its use.[4,5] Pitressin was used because it had been shown to be a powerful coronary vasoconstrictor[6] and might therefore be expected to act detrimentally on the course of infarct healing.

The procedure was essentially similar to that described previously.[1] In all, 47 dogs were used between controls (11), atropine-treated (13), testosterone-treated (11), and pitressin-treated (12) series. Using nembutal anesthesia (25 mg/kilo), the left anterior descending coronary artery and accompanying vein were tied completely in each dog. The animals were permitted to survive 6 weeks before being sacrificed for necropsy examination.

Atropine sulfate‡ was given subcutaneously in doses of 1.3 mg daily for 2 and 6 days per week for the remaining 4 weeks in all but 2 dogs. In these 2 dogs 0.65 mg of atropine was given twice daily for the entire 6-week period. Testosterone propionate§ was given subcutaneously in doses of 25 mg three times weekly for the 6-week period. Pitressin§ was given subcutaneously in doses of 20 pressor units daily for 2 weeks and 6 times weekly for the remaining 4 weeks.

At necropsy all hearts were opened, the ligation of the coronary artery was checked and the endocardial aspect of the infarct was traced on a glass plate. The area was determined by planimeter (*cf.* [1]). The coronary arteries were then injected and X-ray photographs were made in most instances as described previously.[1]

TABLE I.
Effect of Drugs on Infarct Size Calculated as $10^4 \times$ area of infarct (in cm^2) / heart weight (in g)

	Control	Atropine	Testosterone	Pitressin
	639	447	1004	1140
	409	1118	763	619
	933	1026	683	872
	598	980	1815	1006
	922	1168	1075	762
	577	804	592	673
	1123	809	291	865
	1356	815	1099	742
	681	994	101	613
	1000	741	1555	596
	1049	612	102	506
		817		390
		690		
Arithmetic mean	844	851	826	734
Standard deviation	275	186	528	212

The data are summarized in Table I. It will be seen that no alteration in infarct size was produced by the 3 drugs used. The differences in values in each of the series and in their averages are statistically insignificant. It can therefore be concluded that within the experimental error of our method, atropine, testosterone, and pitressin in the manner administered had no demonstrable effect on the healing of experimentally produced myocardial infarcts.

The reported beneficial clinical effect of atropine and of testosterone therefore apparently operates in some way other than by coronary vasodilation. The failure of pitressin to increase infarct size outside the error of the method, suggests the possibility that its pressor effect in these dogs neutralized the coronary vasoconstriction.

It must be borne in mind that this experimental method is relatively crude and that finer differences may be concealed by inescapable variations in technic.

Summary. Atropine, testosterone, and pitressin failed to affect infarct size over a period of 6 weeks following experimental ligation of the left descending coronary artery.

We are indebted to other members of the department for their assistance and to Dr. L. N. Katz for suggesting the problem.

3 Katz, L. N., and Jochim, K., *Am. J. Physiol.*, 1939, **126**, 395.

4 Lesser, M. A., *New Engl. J. Med.*, 1942, **226**, 51.

5 Dock, W. J., *J. Exp. Med.*, 1941, **74**, 177.

6 Katz, L. N., Lindner, E., Weinstein, W., Abramson, D. I., and Jochim, K., *Arch. Int. de Pharm. et de Therap.*, 1938, **59**, 399.

‡ We are indebted to the Abbott Laboratories for the atropine.

§ We are indebted to Schering and Co. for the testosterone and to Parke-Davis and Co. for the pitressin.

15372

The Flow of Blood Supplying the Cardiac Atria.

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Experimental evidence suggests that disturbance of the blood supply of the cardiac atria may lead to auricular fibrillation,[1,2] or to other auricular arrhythmias.[3,4] Aside from anatomical studies, little attention has been directed to the flow of blood to the atrial structures and to factors which may impair that blood supply. These experiments were undertaken in order to study the inflow of blood into one of the atrial arteries, and to note the effect of increased intraatrial tension upon the inflow.

Method. Dogs weighing about 15 kg were anesthetized with veterinary nembutal. In each experiment a Starling heart-lung preparation was set up. The use of the heart-lung preparation permitted closer observation and control of many features of cardiac performance. The largest, and most accessible atrial artery to perfuse was the *left anterior auricular artery*,[5] easily identified as a branch of the circumflex coronary artery at the base of the left auricular appendage. This artery was dissected free; the left circumflex artery was also dissected from its origin for a distance of about 2 cm. A flexible rubber tube, with small glass cannula, was led from the aortic cannula and was inserted into the left circumflex about 2 cm from its origin so as to perfuse the vessel distally. Such perfusion of the circumflex served to maintain myocardial function. The circumflex was ligated at its origin, and a second tube (of metal) was led from the aortic cannula, through the flow-meter, and attached to the proximal segment of the circumflex vessel (as shown in Fig. 1). Arteries arising from this segment, other than the auricular, were securely ligated.

Inflow of blood into the auricular artery was measured, phasically and quantitatively, by the differential pressure orifice-meter devised by Gregg and Green.[6] Calibration of the instrument and interpretation of the flow curves have been described by them, and these were carefully followed in the experiments. The flow curves were synchronized with the cardiac cycle by simultaneous recording either with an electrocardiograph, or with aortic pressure curves using a Wiggers manometer, on the same bromide paper.

Tension within the left auricle was measured by a simple manometer connected into one of the pulmonary veins close to the auricular wall. Estimates of cardiac output were made by collecting outflowing blood in a graduate or siphon-recorder. Twelve experiments were performed on 12 heart-lung preparations showing good function.

Results. For observing auricular arterial inflow under normal conditions, the output of the heart-lung was about 600 cc per minute (commensurate with the capacity of the heart). The blood pressure was adjusted to 100 mm Hg. Tension within the left atrium varied from 3-6 cm of water in different hearts. The venous reservoir was 12 cm above the right auricle.

The normal pattern of inflow into the auricular artery is shown in Fig. 2. The curve indicates that forward flows occur during ventricular systole and diastole. Abrupt interruptions of the stream occur at the onset of ventricular systole; early in ventricular diastole, there is momentary cessation of inflow followed by a short period of backflow.

[1] Resnik, W. H., *J. Clin. Invest.*, 1925, **2**, 125.

[2] Smith, J. R., and Wilson, K. S., *Am. Heart J.*, 1944, **27**, 176.

[3] Cushing, E. H., Feil, H. S., Stanton, E. J., and Wartman, W. B., *Brit. Heart J.*, 1942, **4**, 17.

[4] de Boer, S., *Ergebn. d. Physiol.*, 1923, **21**, 1.

[5] Meek, W. J., Keenan, M., and Theisen, H. J., *Am. Heart J.*, 1928-29, **4**, 591.

[6] Gregg, D. E., and Green, H. D., *Am. J. Physiol.*, 1940, **130**, 144.

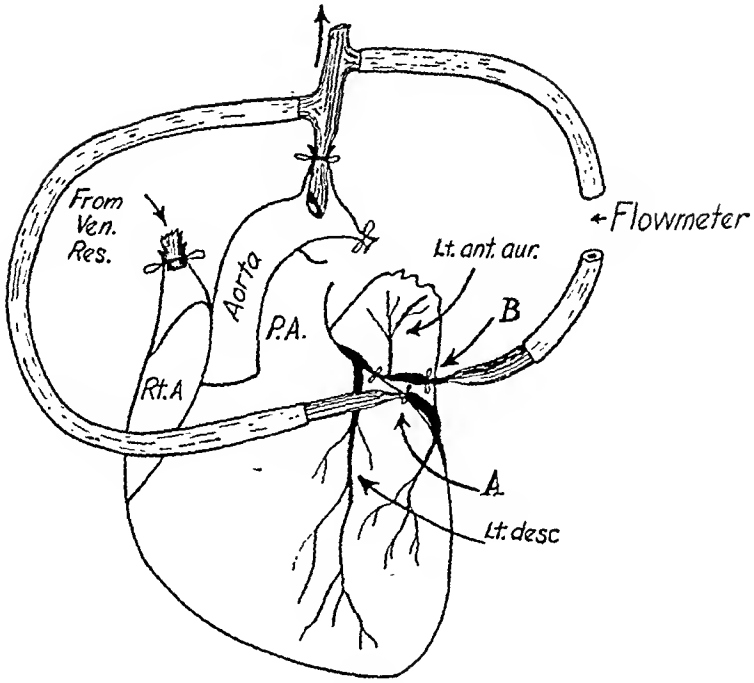

FIG. 1.
Diagram of the arrangement of cannulæ for the perfusion of the left anterior auricular artery. The left circumflex coronary artery is perfused distally (A) to maintain myocardial function. A second system, including the flowmeter, is used to perfuse a proximal segment of the left circumflex (B) of which the auricular artery is a branch.

The peak of rapid inflow in ventricular systole is slightly out of phase with the peak of aortic blood pressure owing, possibly, to delay in the stream flowing through the artificial circuit and flow-meter.

Quantitative analysis of the curves show that in most instances auricular arterial inflow during ventricular systole and diastole are nearly equal, systolic inflow being greater or lesser than that of diastole by a few hundredths cubic centimeter. In these hearts, total inflow for each cardiac cycle varied from 0.08 cc to 0.581 cc. A casual examination of the tracings suggests that the arterial stream during ventricular systole is of greater quantity than in diastole—a discrepancy which is more apparent than real. Rate of inflow in ventricular systole is greater for a short time, producing this form of curve. Tracings of blood inflow into the auricular artery resemble, in general, the metered curves of flow into the principal coronary arteries.[6]

Effect of Raising Left Intra-auricular Tension Upon Atrial Arterial Inflow: Elevation of intra-auricular tension was produced by: 1. induction of cardiac dilatation by raising cardiac output above optimum levels (4 experiments); 2. by inducing anoxia of the preparation (3 experiments), and 3. by producing cardiac dilatation by the administration of 20% chloral hydrate[7] (2 experiments). Cardiac dilatation by any of these methods readily caused a progressive rise of left intra-auricular pressure to 20-25 cm of water. During the period of myocardial dilatation, the blood pressure was maintained at 100 mm Hg, and the cardiac output showed only minor degrees of variation. Heart failure was not permitted to become more severe, for when

[7] Fahr, G., and Buchler, M. S., *Am. Heart J.*, 1943, 25, 211.

TABLE I.
Examples of Quantity of Inflow into the Left Anterior Auricular Artery in 2 Normally Beating Heart-Lung Preparations.

	Cardiac output, cc per min	Blood pressure	Inflow systole, cc	Inflow diastole, cc	Total inflow, cc per cycle
Exp. 7	660	100	0.084	0.072	0.156
" 8	640	100	0.099	0.141	0.240

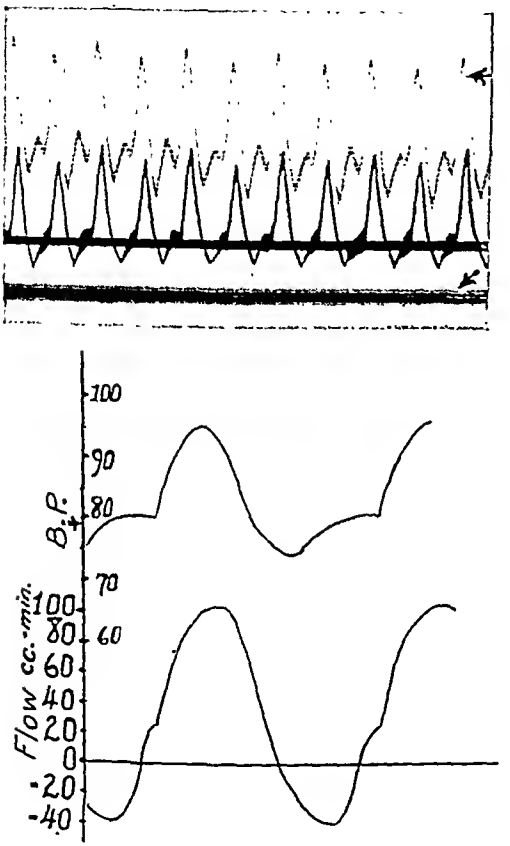

FIG. 2.

Upper: Segment of a curve showing aortic blood pressure tracing (marked by arrow), and the curve of blood inflow into the left anterior auricular artery in a heart-lung preparation. The flow curve has been inked to facilitate reproduction. Black line through the flow curve is line of zero flow; base line for blood pressure is marked by arrow. Lower: Blood pressure and inflow curves redrawn to linear ordinate scales. Analysis of flow curve shows inflow during ventricular systole of 0.249 cc; inflow during ventricular diastole of 0.332 cc. Inflow during cardiac cycle 0.581 cc. Cardiac output was 625 cc per minute.

marked failure occurred blood pressure and output could not be controlled, and the preparation became useless.

TABLE II.
Experiment 10, Normally Beating Heart-Lung Preparation, Blood Pressure Constant at 100 mm Hg. Quantitative Changes in Inflow into Left Auricular Artery During Rise of Left Intra-auricular Tension from Anoxemia.

Left intra-aur. tension, cm water	Cardiac output, cc per min	Aur. art. inflow per cycle, cc
3.3	580	0.140
8.8	640	0.123
24.0	600	0.096
8.8	610	0.136

With blood pressure and cardiac output remaining essentially constant, left auricular arterial inflow decreased as pressure within the auricle rose from myocardial dilatation. Decrease in the quantity flow became apparent as pressure within the auricle rose 8-10 cm of water; diminution of inflow became more marked as auricular tensions increased to 15-20 cm of water (Table II). In general, a rise of intra-auricular tension to 15-20 cm of water, or more, reduced arterial inflow approximately 30-40%. As the heart gradually recovered, the inflow again increased as intra-auricular pressure declined. Analysis of the curves further indicated that the inflow in ventricular systole and diastole was usually uniformly decreased as distension of the auricle occurred. Exceptionally, the inflow during ventricular diastole was greatly curtailed, so that forward flow into the auricular artery took place almost entirely during contraction of the ventricles.

Discussion. These experiments utilized a system conveying blood directly from the aortic cannula of the heart-lung preparation to the left anterior auricular artery. Therefore, the phasic changes observed in the inflow curves depended upon aortic pressure and upon alteration of factors affecting the auricular vascular bed. They were not due to changes of flow in the left circumflex artery.

The pattern of the inflow curves, in the normally beating preparation, suggests that the systolic rise of aortic pressure produces a rapid forward progression of blood in the auricular artery. During ventricular diastole, the flow is less rapid than in systole but is somewhat more sustained. Forward flow is momentarily interrupted at the onset of ventricular systole and diastole, coincident with rapid changes in aortic pressure. Generally there is a sharp backflow from the auricular artery during the rapid fall of aortic blood pressure, early in ventricular diastole.

The diminution of inflow into the auricular artery accompanying elevation of left intraauricular pressure appears to result from changes in the resistance in the auricular vascular bed, since cardiac output and blood pressure were maintained during the period of mild heart failure. It has been suggested that stretching of the cardiac walls may attenuate the intramural vessels, diminishing their capacity.[8] Spalteholz[9] and Unger[10] have shown that the greater part of the venous blood from the left auricle passes by vein to the coronary sinus, but that numerous Thebesian veins exist to account for the escape of a portion of venous blood directly into the auricular cavity. Therefore, a rise of intra-auricular tension may diminish venous outflow from the Thebesian vessels. More probably the distension of the atrial wall exerts pressure upon all of the intramural vessels preventing the free passage of blood through them.

The results of these experiments warrant the suggestion that distension of the auricles, (*e.g.*, resulting from heart failure or valvular disease) may curtail the blood supply to the auricles, favoring the development of auricular fibrillation or other auricular arrhythmias.[2]

Summary. The flow of blood into the left anterior auricular artery was measured phasically and quantitatively in the heart-lung preparation. The curves indicate that forward flow into the auricular artery occurs during ventricular systole and diastole, with abrupt, momentary interruptions of inflow at the onset of ventricular systole and diastole. Elevation of tension within the left auricle diminishes auricular arterial inflow; inflow again increases as pressure within the auricle is restored to normal levels. It is suggested that the interference with auricular blood supply, due to increased intra-auricular tension (as in heart failure) may enhance the establishment of aberrant auricular mechanisms.

8 Vannotti, A., and Blunschy, A., *Z. f. d. ges. exp. Med.*, 1939, **105**, 447.

9 Spalteholz, W., *Anat. Anz.*, 1934, **79**, 212.

10 Unger, K., *Z. f. Anat. u. Entwickl.*, 1937-38, **108**, 356.

15373 P

Pyroninophilic Structures of Liver Cells in Carbon Tetrachloride Poisoning.

A. ROSIN AND L. DOLJANSKI. (Introduced by L. Halberstaedter).

From the Department of Experimental Pathology, The Hebrew University, (Cancer Laboratories), Jerusalem, Palestine.

The present report is concerned with changes in the pyroninophilic structures of the liver cell, as an early effect of carbon tetrachloride poisoning.

In the hepatic cells of various mammals and lower animals a peculiar type of granulation has repeatedly been observed and described.[1] These structures are characteristic in that they stain an intensive red with methyl green-pyronin (Pappenheimer's meth-

1 Krause, R., *Arch. f. mikr. Anat.*, 1893, **42**, 53; Koiransky, E., *Anat. Anz.*, 1904, **25**, 435; Berg, W., *Anat. Anz.*, 1912, **42**, 251; *Arch. f. mikr. Anat.*, 1920, **94**, 518; *Pflüger's Arch. f. d. ges. Physiol.*, 1926, **214**, 243; *Z. f. mikr.-anat. Forsch*, 1927, **12**, 1; *Z. f. mikr.-anat. Forsch.*, 1934, **36**, 87.

od). They vary in size and shape and may have the form of spheres, plump rods or lumps. They are often accumulated in the immediate neighborhood of the nucleus, but occasionally are spread over the entire cytoplasm. There is a clear relationship between the pyroninophilic granule content of the liver cells and the nutritional state of the animal. In livers of fasting animals or in animals fed on carbohydrate or fat only the pyroninophilic structures disappear; in animals kept on a diet rich in proteins and products of protein cleavage they are especially numerous. The pyroninophilic granules can be digested by protein splitting ferments; they give positive Millon's, ninhydrin, diazo, and nitroprusside reactions. These facts led Berg to conclude that the pyroninophilic structures are paraplasmatic accumulations of proteins with a substantial proportion of lower degradation products. According to this author changes in the size and number of pyroninophilic granules are an indication of a disturbed protein metabolism.

The views of Berg were corroborated by a number of investigators.[2-9] Various authors[10-14] have opposed his opinions. Kremer[15] held the pyroninophilic structures to be the products of biliary secretion. The histochemical studies of Brachet[16] and of Biesele[17] made it probable that the structures observed by Berg contain ribonucleic acid as an essential component.

Few studies have been made on pyroninophilic structures under pathological conditions. It has been claimed[18] that in human livers showing cloudy swelling the pyroninophilic material is augmented. In livers of patients dying with severe infections, a marked increase of pyroninophilic granules could be observed.[19]

It has been further noted that administration of adrenalin as well as insulin considerably reduces the amount of pyroninophilic granulations.[3,5,7] Recently Korenchevsky[20] described a decrease in number and size of the pyroninophilic granules in gonadectomized rats, and a return to normal after injection of sex hormones.

In the present investigation we used young albino rats kept on a balanced diet, rich in proteins. Chemically pure carbon tetrachloride was given intraperitoneally in an amount of 0.1 ml per 100 g of body weight. The animals were sacrificed after various intervals. The liver samples were fixed in Zenker's and Carnoy's fluids immediately after removal and embedded in paraffin. The sections, 5 μ thick, were stained with methyl green-pyronin, according to Pappenheimer.

In the liver of normal uninjected rats fed on our standard diet, sufficient in every respect, pyroninophilic structures were invariably present in practically every hepatic cell. They were numerous, of varying size and shape and were fairly evenly distributed throughout the hepatic lobule. The aspect was quite different after administration of carbon tetrachloride. In the animals sacrificed one hour after injection of carbon tetrachloride the pyroninophilic granules in the periphery and the middle zone of the

[2] Cahn-Bronner, C., *Biochem. Z.*, 1914, **66**, 289.

[3] Stübel, H., *Pflüger's Arch. f. d. ges. Physiol.*, 1920, **185**, 74.

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[5] Rothmann, H., *Z. f. d. ges. exp. Med.*, 1924, **40**, 255.

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[7] Paschkis, K., *Klin. Wchnschr.*, 1929, **8**, 1293.

[8] Clara, M., *Z. f. Zellforsch. u. mikr. Anat.*, 1934, **21**, 119.

[9] Li, H. M., *Chinese J. Physiol.*, 1936, **10**, 7.

[10] Levy, M., *Z. f. klin. Med.*, 1924, **98**, 220.

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[13] Sünder, L., *Z. f. mikr.-anat. Forsch.*, 1937, **41**, 541.

[14] Kosterlitz, H. W., and Campbell, R. M., *Nutrition Abstr. and Rev.*, 1945-6, **15**, 1.

[15] Kremer, J., *Z. f. mikr.-anat. Forsch.*, 1933, **33**, 485.

[16] Brachet, J., *C. R. Soc. de biol.*, 1940, **133**, 88.

[17] Biesele, J. J., *Cancer Research*, 1944, **4**, 529.

[18] Never, H. E., *Zentralbl. f. allg. Path. u. path. Anat.*, 1932, **54**, 327.

[19] Santee, F. L., *Bull. Johns Hopkins Hosp.*, 1936, **59**, 427.

[20] Korenchevsky, V., *J. Path. and Bact.*, 1941, **52**, 341.

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15374

Histamine Antagonists. V. Comparison of Benadryl and Pyribenzamine in Histamine and Anaphylactic Shock.

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(Introduced by C. A. Dragstedt).

From the Division of Allergy, Department of Internal Medicine, Northwestern University Medical School, Chicago, Ill.

Following the lead of French investigators, several new antihistaminic compounds have recently been synthesized in this country and made available for experimental and clinical trial. B-dimethylaminoethyl benzhydryl ether (Benadryl) and pyridil-N′-benzyl-N-dimethylethylenediamine (Pyribenzamine), an analogue of the later French compounds, have proven effective in histamine,[1-3] and anaphylactic[3-5] shock and in the management of some allergic conditions in man.[6-10]

In order to have a basis for a comparative activity of these and similar compounds it was felt that an experimental study of these substances by the same technic and in the same laboratory was required. The present experiments deal with the comparative efficacy of Benadryl* and Pyribenzamine* in fatal histamine and anaphylactic shock in guinea pigs.

Histamine Shock. Adult male guinea pigs were given injections of histamine in the dorsal vein of the penis. Histamine phosphate was employed in increasing doses in a series of control animals to determine the 100% lethal dose. (All values of histamine are expressed in terms of the base). Another group of guinea pigs received 3 mg/kg of Benadryl intraperitoneally 15 minutes before the administration of histamine. A third group of animals was similarly prepared with 3 mg/kg of Pyribenzamine (Table I). In the untreated control group, 0.4 mg/kg of histamine resulted in the death of all animals within 5 minutes. At lower doses varying degrees of shock were encountered in the surviving animals. In the Benadryl-treated group significant protection was afforded, in that 2.0 mg/kg of histamine, 5 times the amount necessary to kill all unprotected animals, were required to produce 100% mortality. Some degree of shock was encountered in practically all animals which survived lesser doses. The animals receiving Pyribenzamine showed a considerably higher degree of protection against the lethal effects of histamine. Little evidence of shock and no deaths were observed up to 2.0 mg/kg, while 15.0 mg/kg of histamine were required to kill all animals. The data obtained would indicate that Pyribenzamine is approximately 6 to 7 times more active than Benadryl in preventing fatal histamine shock in guinea pigs.

Anaphylactic Shock. Seventy-two male guinea pigs weighing from 300 to 400 g were passively sensitized by the subcutaneous injection of 0.5 cc of rabbit anti-horse serum

[1] Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharmacol. and Exp. Therap.*, 1945, **83**, 120.

[2] Wells, L. A. Morris, H. C., Bull, H. B., and Dragstedt, C. A., *J. Pharmacol. and Exp. Therap.*, 1945, **85**, 122.

[3] Mayer, R. L., Huttner, C. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

[4] Loew, E. R., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 235.

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[6] Feinberg, S. M., and Friedlaender, S., *J. Allergy*, 1945, **16**, 296.

[7] Curtis, A. C., and Owens, B. B., *Univ. Mich. Hosp. Bull.*, 1945, **11**, 1.

[8] Friedlaender, A. S., *Am. J. Med. Sc.*, 1946, in press.

[9] Friedlaender, S., and Feinberg, S. M., *J. Allergy*, 1946, **17**, 129.

[10] Arbesman, C. E., Koepf, G. F., and Miller, G., *J. Allergy*, 1946, in press.

* Benadryl was supplied by Parke-Davis and Co., Detroit, Mich.; the Pyribenzamine was furnished by Ciba Pharmaceutical Products, Inc., Summit, N.J.

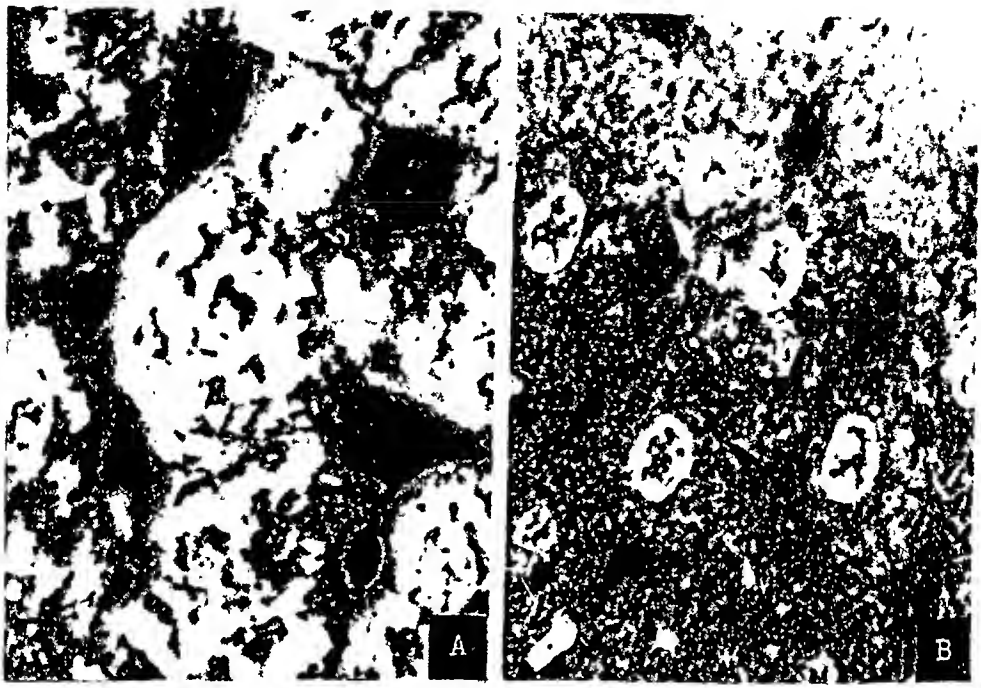

FIG. 1.
Liver of the rat (No. 293) sacrificed 1 hour after the injection of carbon tetrachloride. Fixed in Carnoy's fluid. Stained with methyl green-pyronin.
A. Liver cells from the periphery of the lobule. Mag. × 1900.
B. Liver cells from the central part of the lobule. Mag. × 1500.

lobule were essentially normal in size, distribution and number. In contrast, in the central zone of the lobule the parenchymal cells were free from pyroninophilic material and no traces of red granules could be identified. The line of demarcation between the empty central areas and those containing pyroninophilic granules is sharp. At this period no other noteworthy alterations were observed in the liver parenchym of the treated rats.

Summing up, the administration of carbon tetrachloride, as early as one hour after injection, brings about an alteration of the pyroninophilic structures, leading to their complete disappearance in the hepatic cells in the central part of the lobule. Inasmuch as the nature of the pyroninophilic structures is still a subject of discussion, no conclusive evidence pointing to the significance of these changes can be presented at time. If Berg's concept is correct, the early disappearance of the pyroninophilic granules in liver cells after injection of carbon tetrachloride can be regarded as a proof that disturbed protein metabolism in liver cells is one of the very earliest effects of carbon tetrachloride poisoning.

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From the Division of Allergy, Department of Internal Medicine, Northwestern University Medical School, Chicago, Ill.

Following the lead of French investigators, several new antihistaminic compounds have recently been synthesized in this country and made available for experimental and clinical trial. B-dimethylaminoethyl benzhydryl ether (Benadryl) and pyridil-N'-benzyl-N-dimethylethylenediamine (Pyribenzamine), an analogue of the later French compounds, have proven effective in histamine,[1-3] and anaphylactic[3-5] shock and in the management of some allergic conditions in man.[6-10]

In order to have a basis for a comparative activity of these and similar compounds it was felt that an experimental study of these substances by the same technic and in the same laboratory was required. The present experiments deal with the comparative efficacy of Benadryl* and Pyribenzamine* in fatal histamine and anaphylactic shock in guinea pigs.

Histamine Shock. Adult male guinea pigs were given injections of histamine in the dorsal vein of the penis. Histamine phosphate was employed in increasing doses in a series of control animals to determine the 100% lethal dose. (All values of histamine are expressed in terms of the base). Another group of guinea pigs received 3 mg/kg of Benadryl intraperitoneally 15 minutes before the administration of histamine. A third group of animals was similarly prepared with 3 mg/kg of Pyribenzamine (Table I). In the untreated control group, 0.4 mg/kg of histamine resulted in the death of all animals within 5 minutes. At lower doses varying degrees of shock were encountered in the surviving animals. In the Benadryl-treated group significant protection was afforded, in that 2.0 mg/kg of histamine, 5 times the amount necessary to kill all unprotected animals, were required to produce 100% mortality. Some degree of shock was encountered in practically all animals which survived lesser doses. The animals receiving Pyribenzamine showed a considerably higher degree of protection against the lethal effects of histamine. Little evidence of shock and no deaths were observed up to 2.0 mg/kg, while 15.0 mg/kg of histamine were required to kill all animals. The data obtained would indicate that Pyribenzamine is approximately 6 to 7 times more active than Benadryl in preventing fatal histamine shock in guinea pigs.

Anaphylactic Shock. Seventy-two male guinea pigs weighing from 300 to 400 g were passively sensitized by the subcutaneous injection of 0.5 cc of rabbit anti-horse serum

[1] Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharmacol. and Exp. Therap.*, 1945, **83**, 120.

[2] Wells, L. A. Morris, H. C., Bull, H. B., and Dragstedt, C. A., *J. Pharmacol. and Exp. Therap.*, 1945, **85**, 122.

[3] Mayer, R. L., Huttner, C. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

[4] Loew, E. R., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 235.

[5] Wells, J. A., Morris, H. C., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 104.

[6] Feinberg, S. M., and Friedlaender, S., *J. Allergy*, 1945, **16**, 296.

[7] Curtis, A. C., and Owens, B. B., *Univ. Mich. Hosp. Bull.*, 1945, **11**, 1.

[8] Friedlaender, A. S., *Am. J. Med. Sc.*, 1946, in press.

[9] Friedlaender, S., and Feinberg, S. M., *J. Allergy*, 1946, **17**, 129.

[10] Arbesman, C. E., Koepf, G. F., and Miller, G., *J. Allergy*, 1946, in press.

* Benadryl was supplied by Parke-Davis and Co., Detroit, Mich.; the Pyribenzamine was furnished by Ciba Pharmaceutical Products, Inc., Summit, N.J.

TABLE I.

Protective Effect of Benadryl and Pyribenzamine Against Histamine Shock in Guinea Pigs.

Histamine I.V. mg (base)/kg	Control group Mortality		Group receiving Benadryl 3 mg/kg Mortality		Group receiving Pyribenzamine 3 mg/kg Mortality	
	Total deaths		Total deaths		Total deaths	
	Total used	%	Total used	%	Total used	%
.03- 0.1	0/3	0				
0.2	2/6	33				
0.3	3/6	50				
0.4	10/10	100	0/5	0		
0.8 - 1.6			2/7	29	0/6	0
2.0			2/6	33	0/5	0
2.4 - 3.2			6/6	100	0/4	0
3.6 - 6.8					1/6	16
7 -10					2/10	20
11 -13					3/9	33
15					5/8	62
					10/10	100

TABLE II.

Protective Effect of Benadryl and Pyribenzamine Against Anaphylactic Shock in Guinea Pigs.

Amount of drug used	mg/kg	No. of animals used	Survived	Died
None		12	1	11
Benadryl	1	10	5	5
	2	10	7	3
	3	10	10	0
Pyribenzamine	1	10	4	6
	2	10	7	3
	3	10	10	0

(Table II). After 48 hours, the intravenous injection of 1 cc of horse serum in the penile veins of 12 animals in this group resulted in typical fatal anaphylactic shock in 11. One animal manifested severe symptoms with recovery. The remaining animals were divided into 6 groups of 10 each, and given intraperitoneal injections of 1, 2, or 3 mg/kg of Benadryl or Pyribenzamine 15 minutes before the intravenous administration of 1 cc of normal horse serum. A significant degree of protection was afforded by 1 mg/kg of either drug. Two mg/kg gave somewhat increased protection, while 3 mg/kg of Benadryl or Pyribenzamine protected against fatal anaphylactic shock in all animals tested. Some manifestations of anaphylaxis were observed in the majority of the surviving guinea pigs. Subject to the limitations of the above experiment, this might indicate that there is no essential difference in the anti-anaphylactic activity of the 2 drugs under study.

Discussion. The protective effect of Benadryl and Pyribenzamine against histamine and anaphylactic shock is striking and in accord with the theory that histamine plays a role in anaphylaxis. Pyribenzamine on a weight basis has a greater protective effect against histamine than does Benadryl as manifested by the large increase in the LD_{100} of histamine in Pyribenzamine-treated animals. On a weight basis the 2 drugs appear to have an equal effectiveness against anaphylactic shock. This apparent discrepancy may be due to the fact that the maximum amount of histamine liberated during anaphylaxis in the guinea pig is of the order of 0.4 mg/kg, at which dose the 2 drugs are nearly equally effective. There is of course the consideration that phenomena other than the liberation of histamine may account for some difference between the protective effect of chemical agents against histamine shock on the one hand, and against anaphylactic shock on the other.

Summary. The LD_{100} of histamine was

first determined in a control group of guinea pigs. It was found that approximately 5 times this amount was required to kill all animals previously treated with 3 mg/kg of Benadryl, while 35 times the lethal dose of histamine was necessary to produce 100% mortality in animals receiving 3 mg/kg of Pyribenzamine. No apparent difference was discernible between the 2 drugs in preventing anaphylaxis in passively sensitized guinea pigs. One mg/kg of either compound gave significant protection against a shocking dose of antigen, while 3.0 mg/kg prevented fatal anaphylaxis in all animals tested.

15375

Bed-side Agglutination Test with Whole Blood for Rapid Diagnosis of Tularemia.*

RAÚL M. TOVAR. (Introduced by M. R. Castaneda).

From the Department of Medical Research, General Hospital, Mexico D. F.

The agglutination test has been the most practical method for the laboratory diagnosis of tularemia. McCoy and Chapin[1] showed the presence of agglutinins in the serum of patients infected with *B. tularense* and Francis[2] applied the agglutination test to the serological diagnosis of the infection. Recently Damond and Johnson[3] described what they call the "shake method" of agglutination by which it is possible to accelerate the reaction and read the results in 3 minutes.

Considering the possibility of a further improvement by using the method recommended by Castaneda and collaborators for typhus fever[4] and brucellosis,[5] we prepared a concentrated antigen conveniently stained and used either with whole blood as a bed-side test or with serum as in the case of the so-called rapid antigens developed by Huddleson[6] and Welch.[7]

Preparation of the Antigen. The strain of *B. tularense* No. 408, obtained by courtesy of Dr. R. R. Parker from the Rocky Mountain Laboratory of Hamilton, Montana, was used for the preparation of the antigen. The culture medium, recently described,[8] consisted briefly in a concentrated liver infusion with cystine, glucose, sodium chloride, peptone and agar, without blood or hemoglobin and distributed in Roux's bottles. Each bottle was inoculated with a concentrated emulsion of *B. tularense* and after 72 hours of incubation at 37°C the organisms were emulsified with isotonic saline containing 10% formaline (40%), filtered through wet cotton and left at ordinary temperature for 72 hours. The emulsion was centrifuged and the supernatant fluid was discarded; the organisms were emulsified in a small amount of isotonic saline. The concentration of the emulsion was standardized in order that one-tenth of antigen diluted with 10 cc of saline gave a turbidity corresponding to No. 3 of McFarland's Nephelometer. When the concentration of the antigen was adequate, enough aqueous solution of methylene blue was added to stain the antigen to a deep blue color. After 24 hours the antigen was centrifuged at high speed and the supernatant fluid was discarded.

* This work was aided by grants from the University of Mexico and Eli Lilly Co. of Indianapolis, Indiana.

1 McCoy, G. W., and Chapin, C. W., *J. Infect. Dis.*, 1912, **10**, 61.

2 Francis, E., *Medicine*, 1928, **7**, 411.

3 Damond, S. R., and Johnson, M. B., *J. Lab. and Clin. Med.*, 1944, **29**, 976.

4 Castaneda, M. R., Silva, R. G., and Monnier, A., *Rev. Med. del Hosp. General*, 1940, **8**, 382.

5 Castaneda, M. R., "Brucelosis," First edition, *Medicina*, 1942.

6 Huddleson, I. F., Tech. Bull. No. 123, Mich. Agric. Exp. Station, 1932.

7 Welch, H., and Stuart, C. A., *J. Lab. and Clin. Med.*, 1936, **21**, 411.

8 Tovar, R. M., *Rev. del Inst. de Salubridad y Enf. Trop.*, 1945, **6**, 181.

The stained organisms were emulsified in a small amount of isotonic sodium citrate solution (1.1%), containing merthiolate to a concentration of 1:5000.

Samples of the concentrated material are diluted to various proportions with isotonic citrate and each dilution tested for specificity and sensitivity using samples of serums with titers previously determined by the tube agglutination method. The test is performed mixing a drop of antigen with a drop of serum in a slide, and the sample giving definite agglutination within one minute with a serum of 1:50 titer is selected as a basis for further titrations. Then the antigen is tested for sensitivity which is made comparing the titer of a serum determined by the standard tube agglutination method and the results of tests performed on a glass slide using the rapid antigen following the method of Huddleson for brucellosis.[6]

The specificity of the antigen has been determined with a few available sera from patients suffering from tularemia, 2 from Mexico and 4 obtained by courtesy of Dr. Parker (Hamilton, Montana); all of them were confirmed cases. We also used sera from guinea pigs and rabbits experimentally infected with a non-virulent *B. tularense* strain. The inactivity of the antigen when mixed with normal sera or with serum from persons suffering from various infections, not including tularemia or brucellosis, was demonstrated performing 1600 comparative tests using the rapid antigen and the tube agglutination method.

When the antigen is found satisfactory it is submitted to tests with whole blood of guinea pigs or rabbits infected with *B. tularense*.

Technic of the test. Bed-side test with whole blood. The agglutination test with whole blood is performed at the bed side, mixing on a clean slide the antigen with blood taken by puncture of the ear or from the finger. The amount of antigen is that carried by a wire loop of 4 mm diameter and that of the blood is what is taken with a 2 mm loop. The slide is moved to and fro and the effect observed in front of a window or a bright light. The positive test appears within one minute, consisting of a definite separation of colors (blue of the antigen and red of the blood) and the immediate formation of clumps of antigen, which because of the rotation of the mixture, have a tendency to be accumulated in the periphery forming a blue ring. In the positive reactions there are different intensities that we called 1_t, 2_t, 3_t and 4_t, according to the time of clumping and size of clumps of antigen; they are related to a low or high agglutinating titer of the blood serum. The rapid reaction with whole blood is positive only in cases in which the agglutinating titer of the serum is above 1:100. In the negative tests there is neither color separation nor clumping of the antigen, the mixture remains homogeneous until drying.

The spontaneous clumping of red blood cells may be confused with a positive result for which it is necessary to perform a new test using the blood serum.

Rapid test with blood serum. The antigen has been satisfactory, used as a screen test, mixing a droplet of antigen with an equal amount of serum. When the test is positive there is an immediate formation of blue clumps of antigen and if it is negative the mixture remains homogeneous until drying. The screen test is positive when the agglutinating titer of the serum is above 1:20. The screen test with serum is particularly useful to pick up suspicious cases when a considerable number of samples is submitted to the laboratory. The positive serums are further tested by the rapid method following the technic of Huddleson and finally by tube agglutination using suitable emulsions of *B. tularense* to determine the agglutinating titer.

Results. The bed-side test with whole blood was performed with known cases of tularemia, suspicious cases detected by previous agglutination tests and blood of animals experimentally infected. The negative controls were normal persons or patients suffering from various diseases. We included a group of 100 cases of brucellosis, which according to Francis and Evans[9] produces a high percentage of cross-agglutination with *B. tularense*. Table I

[9] Francis, E., and Evans, A. C., *Pub. Health Rep.*, 1926, 41, 1273.

TABLE I.
Bed-side Agglutination Test with Rapid *B. tularense* Antigen and Whole Blood.

No. of cases	History	Positive tests	%
100	Normal adults	0	0
100	Adults suffering from various infectious diseases	0	0
100	Brucellosis patients	10	10
30	Serological reactors to *B. tularense**	30	100
2	Confirmed cases of tularemia	2	100
20	Guinea pigs and rabbits experimentally infected	20	100

* Reactors determined by tube agglutination tests.

summarizes the results of the tests.

It may be seen that in spite of the few positive cases, the results of the test were very significant. The test was repeated many times in each case. The positive tests were clear cut in the 30 persons in whom it was found that the serum had a significant titer of agglutinins for *B. tularense*. In regard to the 10 cases of cross-agglutination found in the group of 100 patients suffering from brucellosis this is not surprising because of the previous findings referred to above. These cases of cross-agglutination can be readily differentiated by means of selective agglutination reported elsewhere.[10]

In the guinea pigs experimentally infected with tularemia the test was positive after the 7th day of inoculation and remaining positive during 7 months until discarded.

All individuals showing a positive bed-side agglutination test with whole blood, including the 10 cases infected with *Br. melitensis*, gave allergic skin reaction when injected by intradermal route with "Tulargen,"[10] an extract obtained by grinding *B. tularense*. The opsonocytophagic test performed with killed *B. tularense* as antigen and blood of the same persons, was positive in all cases.

Summary. A bed-side test for the rapid serological diagnosis of tularemia is described. The test is performed mixing a droplet of a concentrated suspension of *B. tularense* with a droplet of whole blood taken from the ear or from the finger of the patient. The minimum agglutinative titer of the patient's serum must be 1:100 to be detected by the rapid test. The antigen may be used for screen tests with blood serum.

[10] Tovar, R. M., *Medicina*, 1945, **25**, 331.

15376

Some Pharmacological Properties of the Monoanilide of Aconitic Acid.

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The monoanilide of aconitic acid was prepared by one of us* from the *cis* anhydride according to the method of Nau, *et al.*[1] This compound was one of a series of derivatives of aconitic acid which were being tested as nonaqueous solvents for medicinal agents. The anilide group is attached to one of the carboxyl groups connected to the unsaturated carbon linkage. It was deemed of interest to compare the toxicity and the analgesic and antipyretic actions of this compound with those of acetanilide.

Toxicity. Forty rats weighing approxi-

* Emil K. Ventre.

[1] Nau, C. A., Brown, E. B., and Bailey, J. R., *J. A. C. S.*, 1925, **47**, 2596.

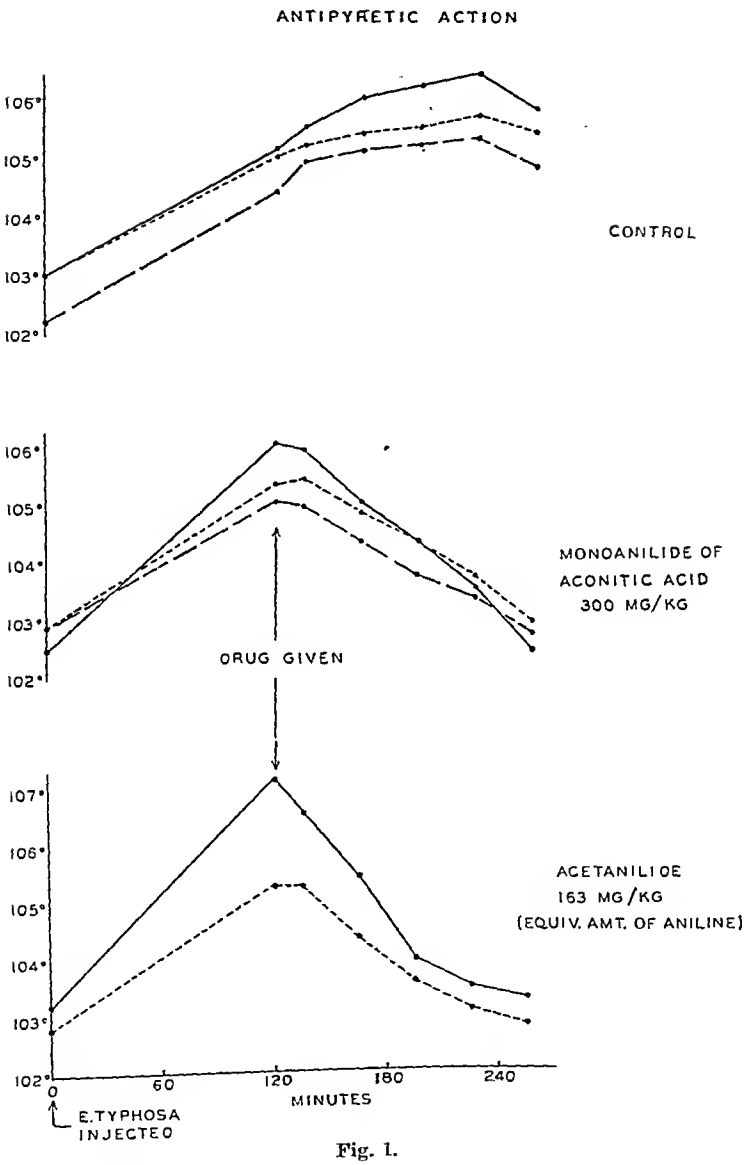

Fig. 1.

mately 200 g each were used in determining the LD_{50} of the compound when administered in suspension through a stomach tube. Twenty of the rats were used in a preliminary series of experiments to learn the approximate LD_{50} and the other 20 were divided into groups of 4 for each of 5 dose levels between 3.8 g/kg and 4.2 g/kg. The data in Table I support the assumptions that the toxicities of the monoanilide of aconitic acid and acetanilide are due primarily to the aniline in the molecules and that the 2 compounds have similar toxicities on a molecular basis.

Analgesic Action in Dogs. Three dogs were given on different days oral doses of 50 mg./kg of the monoanilide of aconitic acid and 27.25 mg/kg of acetanilide—amounts having equal weights of aniline. The rise in pain threshold was measured in each case

TABLE I.
Toxicity.

Compound	LD_{50}	Aniline %	Aniline g
Monoanilide of aconitic acid	4.0	37	1.5
Acetanilide	2.42	68	1.6

by the method of Hardy, *et al.*,[3] as adapted for use with animals. The data in Table II show that these 2 compounds in the doses given were essentially of equal efficiency in raising the pain threshold of dogs.

TABLE II.
Analgesic Action

	Rise in pain threshold			
	Monoanilide		Acetanilide	
Dog No.	Peak rise, %	Time, min	Peak rise, %	Time, min
1	17	50	20	65
2	18	55	18	60
3	17	60	16	60

[2] Sollman, T. H., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, J. W. Stacey Co., San Francisco, 1939, 241.

[3] Hardy, J. D., Wolff, H. G., and Goodell, H., *J. Clin. Invest.*, 1940, **19**, 649.

Antipyretic Action in Rabbits. An elevation in body temperature was produced in 8 rabbits by injecting intravenously 1 cc of a preparation of killed *Eberthella typhosa* containing approximately 10^7 organisms/cc. Rectal temperatures were measured before the injection and at intervals afterward. Either the monoanilide of aconitic acid or acetanilide was administered orally to groups of rabbits after an elevation of 2-4 degrees in body temperature was noted, usually 2 hours after the initial injection. The resultant decreases in temperature are compared in Fig. 1 with the temperatures of a group of rabbits given no drug.

Summary. The monoanilide of aconitic acid appears to have no advantages over acetanilide as an analgesic or antipyretic agent. The activity of the monoanilide seems to be related to the aniline in the molecule, as is the case with acetanilide.[4] No additional pharmacological actions were noted which could be ascribed to the propene group of the aconitic acid.

[4] Michel, H. O., Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Therap.*, 1937, **61**, 321.

15377

Starch Reaction as Aid in Identification of Causative Agent of "European Blastomycosis."

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The clinical picture and symptomatology of "European Blastomycosis" ("Torulosis") is variable, and unreliable as a basis of diagnosis. The differentiation of the etiological agent is beset with difficulties, and its cultural and morphological resemblance to contaminating non-pathogenic yeasts has given rise to much confusion in the literature of yeast infections. The diagnostic scheme outlined below may be helpful in this connection, especially to pathologists without training in mycology, as it avoids the use of refined morphological criteria.

While studying the biochemical properties of nonfermenting capsulated yeasts, we observed that a strain isolated from a case of torula-meningitis produces extracellular starch in special growth conditions.[1] The amount of starch produced is considerable and can easily be detected macroscopically when a plate culture of the organism is flooded with Lugol's iodine solution.

We have now extended our investigation

[1] Aschner, M., Mager, J., and Leibowitz, J., *Nature*, 1945, **156**, 295.

to 16 other strains of this pathogenic yeast and in all of them starch formation was observed in the growth conditions indicated below. The individual strains varied as to the intensity of starch production. In the majority of cases a positive reaction for starch could be obtained after 24 - 48 hours of growth. In some strains the formation of starch was weaker and delayed for 5-8 days. A large collection of other yeast genera was tested and none of them exhibited the phenomenon of extracellular starch production. A substance which stains blue with iodine may be found in cultures of Schizosaccharomyces,[2] but only intracellularly and in very limited amount. The production of extracellular starch seems to be specific for a group of nonfermenting capsulated yeasts of which *Torulopsis neoformans* is a representative. This group includes also nonpathogenic species[3] which can, however, be differentiated by testing the growth optimum. Benham has shown, that whereas pathogenic *Torulopsis neoformans* (*Cryptococcus hominis*, according to Benham's nomenclature) thrives at 37°C, nonpathogenic species of this group show optimum growth at 30°C and poor or no growth at 37°C.[2]

In the light of these findings we arrive at the following method for the identification of *Torulopsis neoformans:*

Yeasts isolated from cases suspected of blastomycosis should be transferred to a synthetic medium composed as follows:

$(NH_4)_2SO_4$	0.1%
$MgSO_4$	0.05%
KH_2PO_4	0.1%
Glucose	1%
Thiamine	0.2 μg per ml
Agar-agar	2.5%

Ammonium sulfate serves in this medium both as a source of nitrogen and as a regulator of the acidity necessary for production of starch.[1] Vitamins other than thiamine are not essential for cultivation of starch-producing yeasts in synthetic media.

After some days of incubation at 37°C, the plate is flooded with Lugol's iodine solution. In positive cases the whole streak of growth usually turns a deep blue. If, however, the amount of starch elaborated by the yeast is small, the reaction is perceptible only in isolated spots and after excess of iodine has disappeared. The extracellular character of the starch may be ascertained by examining with iodine the color of the agar beneath a growth-streak after the cells are scraped off from the agar surface. An alternative method is to cultivate the yeast in a liquid medium (composed as above), where optimal growth can be obtained by aeration or continuous shaking. After the cells have been separated by centrifugation, the supernatant is tested for starch.

According to our results, ability of extracellular starch formation combined with unimpaired growth between 37° and 40°C are features characteristic enough to classify a nonfermenting yeast as *Torulopsis neoformans* (Sanfelice) Lodder.[4,5]

Summary. A method for the identification of *Torulopsis neoformans* (syn. *Torula histolytica, Cryptococcus hominis*) is described.

The method is based on the ability of this organism to produce extracellular starch in special growth conditions.

We should like to express our thanks to Prof. Kluyver, Dr. Lodder, and Dr. Benham for their kindness in sending us a large number of different yeast cultures.

2 Beijerinek, M. W., *Centralblatt f. Bakt. u. Parasitenk.*, 1897, **3**, 449.

3 Benham, R. W., *J. Infect. Dis.*, 1935, **57**, 255.

4 Lodder, J., 1934, *Die Anascosporogenen Hefen*, p. 152, Amsterdam.

5 Lodder, J., *Mycopathologia*, 1938-9, **1**, 62.

15378

Histochemical Distribution of Alkaline Phosphatase in Dog Liver After Experimental Biliary Obstruction.

M. WACHSTEIN AND F. G. ZAK. (Introduced by E. P. Pick).

From the Laboratories of Mount Sinai Hospital, New York City.

Gomori[1] was the first to notice that the bile capillaries in the liver of some species showed marked phosphatase activity. The constant increase of serum alkaline phosphatase after ligation of the bile ducts in the dog[2] and the fairly regular phosphatase activity of the bile capillaries in the dog liver suggested that a study be made of the behavior of alkaline phosphatase activity in the liver after ligation of the bile ducts.

Material and Methods. In 9 mongrel dogs the common bile duct was ligated under nembutal anesthesia after an initial biopsy had been taken. In 2 acute experiments the cystic duct also was ligated. Serum phosphatase activity was estimated with Bodansky's method[3] using a Leitz photocolorimeter. Gomori's method[4] as modified by Kabat and Furth[5] was used for the histochemical demonstration of alkaline phosphatase activity. Alternate sections were routinely incubated for 2 and 14 hours. When indicated, the incubation period was shortened to 30 and 60 minutes. No counterstain was employed.

Results. The changes in the dog liver after duct ligation as seen in slides stained with hematoxylin-eosin, were essentially similar to those previously described.[6,7] They consisted of hyperemia, atrophy of liver cells in the central fields, occurrence of bile thrombi, predominantly in the central fields and very occasional occurrence of small foci of necrosis. Bile pigment was seen in the liver cells mainly around central fields. Kupffer cells frequently contained bile pigment.

Normal Liver. (2-hour incubation). With the exception of bile capillaries and larger bile ducts, there was only very faint phosphatase activity in all other structures. The cytoplasm of the liver cells took on a faint grayish color. Most of the nuclei could be recognized due to some staining of the nuclear membrane and of the nucleoli. Only occasionally was there activity in the endothelium of the sinusoids near the periportal areas. Larger bile ducts in the periportal areas showed marked phosphatase activity. The bile capillaries were outlined as fine, black, lines sometimes showing a definite lumen (Fig. 1). They showed frequently, the well-known tortuous appearance. In some areas, they were seen between 2 liver cell cords (trabecular), in others they formed networks apparently surrounding single liver cells. From the trabecular capillaries intercellular branches went off at various angles. On cross section, they appeared as small round black dots or circles. Very occasionally, the transition of a capillary into a bile duct could be seen. The activity of the bile capillaries was somewhat more distinct near the periportal fields than around central areas. There was, however, some variation in different livers.

Normal Liver. (14-hour incubation). The staining of the cytoplasm and of the nuclei became more intense. The activity of the sinusoidal lining as well as of their nuclei became marked. Connective tissue cells and occasional mononuclear cells in the periportal fields showed also some activity. The intensification of the reaction was moderate in

[1] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[2] Armstrong, A. R., King, E. F., and Harris, R. I., *Canad. M. A. J.*, 1934, **31**, 14.

[3] Bodansky, A., *Am. J. Clin. Path.*, Techn. Suppl., 1937, **1**, 51.

[4] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[5] Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

[6] Ogata, T., *Beitr. Z. Path. Anat. u. Z. Allgem. Pathol.*, 1913, **55**, 236.

[7] Hiyeda, D., *Beitr. Z. Path. Anat. u. Z. Allgem. Pathol.*, 1925, **73**, 541.

to 16 other strains of this pathogenic yeast and in all of them starch formation was observed in the growth conditions indicated below. The individual strains varied as to the intensity of starch production. In the majority of cases a positive reaction for starch could be obtained after 24 - 48 hours of growth. In some strains the formation of starch was weaker and delayed for 5-8 days. A large collection of other yeast genera was tested and none of them exhibited the phenomenon of extracellular starch production. A substance which stains blue with iodine may be found in cultures of Schizosaccharomyces,[2] but only intracellularly and in very limited amount. The production of extracellular starch seems to be specific for a group of nonfermenting capsulated yeasts of which *Torulopsis neoformans* is a representative. This group includes also nonpathogenic species[3] which can, however, be differentiated by testing the growth optimum. Benham has shown, that whereas pathogenic *Torulopsis neoformans* (*Cryptococcus hominis*, according to Benham's nomenclature) thrives at 37°C, nonpathogenic species of this group show optimum growth at 30°C and poor or no growth at 37°C.[2]

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The method is based on the ability of this organism to produce extracellular starch in special growth conditions.

We should like to express our thanks to Prof. Kluyver, Dr. Lodder, and Dr. Benham for their kindness in sending us a large number of different yeast cultures.

2 Beijerinck, M. W., *Centralblatt f. Bakt. u. Parasitenk.*, 1897, **3**, 449.

3 Benham, R. W., *J. Infect. Dis.*, 1935, **57**, 255.

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15378

Histochemical Distribution of Alkaline Phosphatase in Dog Liver After Experimental Biliary Obstruction.

M. WACHSTEIN AND F. G. ZAK. (Introduced by E. P. Pick).

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Gomori[1] was the first to notice that the bile capillaries in the liver of some species showed marked phosphatase activity. The constant increase of serum alkaline phosphatase after ligation of the bile ducts in the dog[2] and the fairly regular phosphatase activity of the bile capillaries in the dog liver suggested that a study be made of the behavior of alkaline phosphatase activity in the liver after ligation of the bile ducts.

Material and Methods. In 9 mongrel dogs the common bile duct was ligated under nembutal anesthesia after an initial biopsy had been taken. In 2 acute experiments the cystic duct also was ligated. Serum phosphatase activity was estimated with Bodansky's method[3] using a Leitz photocolorimeter. Gomori's method[4] as modified by Kabat and Furth[5] was used for the histochemical demonstration of alkaline phosphatase activity. Alternate sections were routinely incubated for 2 and 14 hours. When indicated, the incubation period was shortened to 30 and 60 minutes. No counterstain was employed.

Results. The changes in the dog liver after duct ligation as seen in slides stained with hematoxylin-eosin, were essentially similar to those previously described.[6,7] They consisted of hyperemia, atrophy of liver cells in the central fields, occurrence of bile thrombi, predominantly in the central fields and very occasional occurrence of small foci of necrosis. Bile pigment was seen in the liver cells mainly around central fields. Kupffer cells frequently contained bile pigment.

Normal Liver. (2-hour incubation). With the exception of bile capillaries and larger bile ducts, there was only very faint phosphatase activity in all other structures. The cytoplasm of the liver cells took on a faint grayish color. Most of the nuclei could be recognized due to some staining of the nuclear membrane and of the nucleoli. Only occasionally was there activity in the endothelium of the sinusoids near the periportal areas. Larger bile ducts in the periportal areas showed marked phosphatase activity. The bile capillaries were outlined as fine, black, lines sometimes showing a definite lumen (Fig. 1). They showed frequently, the well-known tortuous appearance. In some areas, they were seen between 2 liver cell cords (trabecular), in others they formed networks apparently surrounding single liver cells. From the trabecular capillaries intercellular branches went off at various angles. On cross section, they appeared as small round black dots or circles. Very occasionally, the transition of a capillary into a bile duct could be seen. The activity of the bile capillaries was somewhat more distinct near the periportal fields than around central areas. There was, however, some variation in different livers.

Normal Liver. (14-hour incubation). The staining of the cytoplasm and of the nuclei became more intense. The activity of the sinusoidal lining as well as of their nuclei became marked. Connective tissue cells and occasional mononuclear cells in the periportal fields showed also some activity. The intensification of the reaction was moderate in

[1] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[2] Armstrong, A. R., King, E. F., and Harris, R. I., *Canad. M. A. J.*, 1934, **31**, 14.

[3] Bodansky, A., *Am. J. Clin. Path.*, Techn. Suppl., 1937, **1**, 51.

[4] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[5] Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

[6] Ogata, T., *Beitr. Z. Path. Anat. u. Z. Allgem. Pathol.*, 1913, **55**, 236.

[7] Hiyeda, D., *Beitr. Z. Path. Anat. u. Z. Allgem. Pathol.*, 1925, **73**, 541.

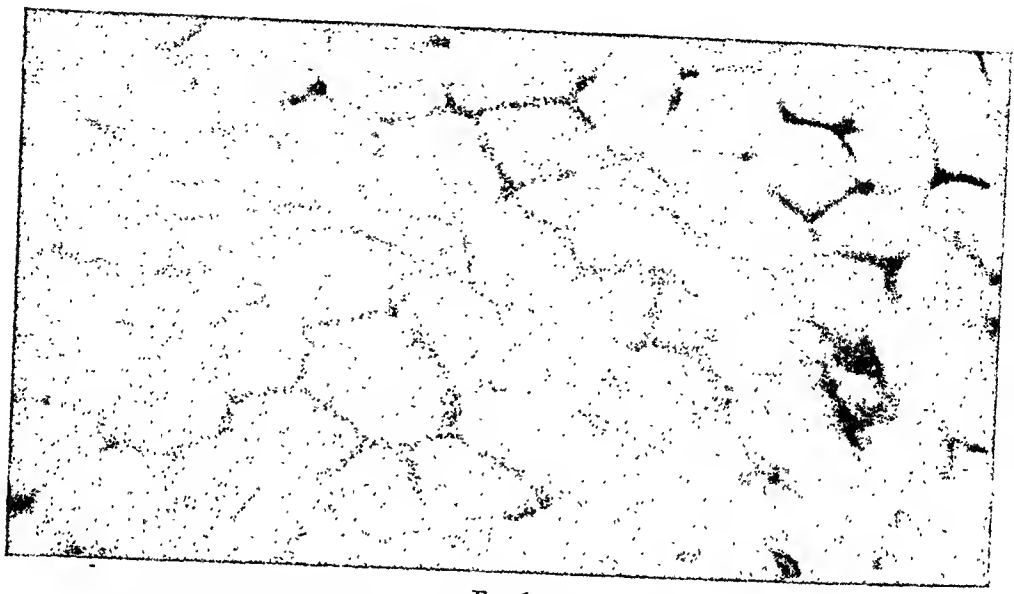

FIG. 1.
Alkaline phosphatase activity in the normal dog liver. The bile capillaries show prominent staining. Incubation in the substrate mixture for 2 hrs. × 800.

some, quite marked in other livers. In the latter group, the bile capillaries also showed stronger staining and occasionally their outline became hazy. The concomitant staining of the other structures made the activity of the bile capillaries less conspicuous.

Effect of Duct Ligation. (3-4 days). Two dogs died after 3-4 days because of extensive wound infection. One was killed 4 days after ligation. This dog showed a rise of blood serum phosphatase from 2.4 to 43.2 units. In sections incubated for 2 hours a considerable number of bile capillaries were wider than normal. Some showed a hazy outline. There was some increase in cytoplasmic phosphatase. After longer incubation, this increase became more striking and the cytoplasm showed many fine, black granules. The 2 dogs that died showed very marked dilatation of the bile capillaries which were considerably widened and not clearly demarcated, from the surrounding cytoplasm. In addition, considerable increase in cytoplasmic activity was seen.

One Week. One dog was killed after 8 days. Serum phosphatase rose from 1.1 to 47.6 units. In sections incubated for 2 hours, there was very marked dilatation of the bile capillaries which stood out much clearer than in the biopsy. Many of these showed a distinct lumen. The demarcation from the cytoplasm was not sharp in a large number of these capillaries. The cytoplasm showed increase in activity somewhat more pronounced around the periportal fields. The bile ducts in the periportal fields showed marked increase in activity and their lumen was filled by intensely dark staining material. After longer incubation, the reaction became so intense that the structures could hardly be differentiated.

Two Weeks. Two dogs were killed after 2 weeks. In one the serum phosphatase rose from 1 to 36 units. In this dog, dilatation of the bile capillaries occurred predominantly at the periportal fields. The second dog showed very widespread dilatation of the bile capillaries. While some of them still showed their clear outline, many became very irregularly demarcated and hazy. The increase in cytoplasmic activity was very conspicuous.

Three Weeks. One dog was killed 3 weeks after ligation. His initial serum phosphatase of 1.4 units rose to 27.6 units in 8 days, and to 32.2 units in 14 days. The increase in phosphatase activity was so intense that sections incubated longer than one hour could

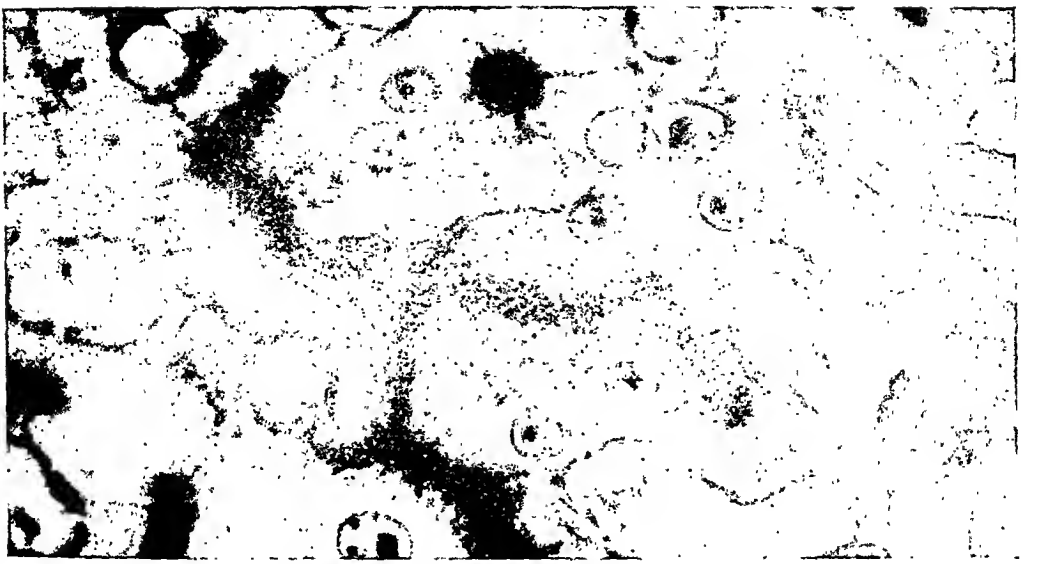

FIG. 2.
Alkaline phosphatase activity in the liver of a dog, 3 weeks after ligation of the common bile duct. Conspicuous widening of many bile capillaries. Incubation in the substrate mixture for 30 minutes. ×800.

not be used. Widening of the bile capillaries was extremely marked in many areas (Fig. 2). It appeared as if large amounts of phosphatase were accumulated in liver cells near the bile capillaries. In this way, the width of the bile capillaries, on cross section, appeared to be 4 to 5 times larger than normal. Occasionally, a lumen could be made out. In contrast to the conspicuous changes in many of the capillaries, some were of fairly normal appearance.

Acute Experiments. In 2 dogs after initial biopsy, the cystic and common ducts were ligated and biopsies were taken after 30, 60, 90, and 120 minutes. No changes were seen in serum phosphatase in one of the dogs in which this examination was carried out. After one hour the bile capillaries stood out somewhat more distinctly and showed occasional light dilatation. There was also a very slight increase in cytoplasmic activity.

Comment. The source of the increased amount of alkaline serum phosphatase which occurs in obstructive and to a lesser degree in parenchymatous jaundice is still controversial.

The accumulation of phosphatase around bile capillaries is in marked contrast to the diffuse increase of enzymatic activity which occurs in the liver of protein depleted mice and rats and to a lesser degree in starvation.[8] It can best be interpreted as retention of alkaline phosphatase in liver cells caused by external obstruction. No significant increase in alkaline phosphatase activity is seen, on the other hand, in necrotic liver cells.[8] The behavior of the histochemically demonstrable phosphatase activity under various experimental conditions therefore, supports the opinion of those who believe[9,10] that the increase of serum phosphatase in liver damage is due to disturbed excretion but not to increased production of alkaline phosphatase in the liver cells.[11]

Summary. Bile capillaries show distinct phosphatase activity in the normal dog liver. This property can be used for their microscopic demonstration. After ligation of the common bile duct there is not only marked

[8] Wachstein, M., *Arch. Path.*, 1945, **40**, 57.

[9] Armstrong, A. R., and Banting, F. G., *Canad. M. A. J.*, 1935, **33**, 243.

[10] Maddock, S., Schmidt, G., and Thannhouser, S. F., *Federation Proc.*, 1942, **1**, 181.

[11] Bodansky, A., *Enzymologia*, 1937, **3**, 258; PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 800.

dilatation of the bile capillaries but also apposition of phosphatase around them which increases with the duration of the experiment. The changes in the distribution of alkaline phosphatase in histochemical preparations favor the assumption that the increase of serum phosphatase in liver damage is due to the inability of the liver cells to excrete the enzyme rather than to increased production in the liver itself.

15379

Morphologic Effects of DDT on Nerve Endings, Neurosomes, and Fiber Types in Voluntary Muscles.*

EBEN J. CAREY, ESTELLE M. DOWNER, FRANCES B. TOOMEY, AND EUGENE HAUSHALTER.

From the Department of Anatomy, Marquette University School of Medicine, Milwaukee, Wisc.

The effect of DDT (2,2 bis(p-chlorophenyl)-1,1,1-trichloroethane) on the voluntary neuromuscular apparatus in the living, intact animal is unknown. Lewis and Richards[1] state that DDT does not affect the growth of various cells in 7- to 8-day chick embryos and in a 1-day rat in hanging drop cultures. They are unable to explain the apparent paradox of the toxicity of DDT to intact, living animals and its non-toxicity on their isolated cells in hanging drop cultures. Yeager and Munson[2] have presented physiological evidence for a possible site of action of DDT in bringing about repetitive discharges of nerve impulses into the muscles of the roach, *Periplaneta americana*, which indicates that the site (or sites) referred to is that region of a nerve which lies between the origin of its fibers in the ventral nerve cord and the termination of its fibers in the leg, exclusive of the origin and the endings, that is, the myoneural junctions. Their results suggest that DDT can act more readily on motor than on sensory nerves and, further, that DDT stimulates motor nerves somewhere along their course between the cells of origin in the ventral nerve cord and the nerve endings in muscle.

The peripheral effects of DDT on the living, nerve-intact muscle, nevertheless, are visibly evident in chameleons and rats, in the persistent, involuntary, clonic contractions and occasional epileptoid convulsions and violent tremors of the entire voluntary musculature until complete flaccid or spastic paralysis and death occur. The purpose of this paper, therefore, is to demonstrate the striking morphologic changes observed in the voluntary neuromuscular apparatus of chameleons and rats during the transmission of a quantitative increase of neurogenic substances into the myoplasm of some muscle fibers affected by DDT.

Methods. A quick-killing emulsion[1] was prepared which contained 1% DDT, 8% olive oil, 1% gum arabic, and 90% Locke's solution; 2 cc of this emulsion was injected intraperitoneally into 48 white rats *(Mus norvegicus)*, average weight 250 g, and 0.1 cc into 100 summer pseudo-chameleons *(Anolis caroliniensis)*, average weight 3 to 8 g. Eight rats were selected at 12-hour intervals after the injection of DDT for microscopic study of the gastrocnemius muscle, sciatic nerve, and spinal cord. Various histologic technics were employed for the study of Nissl substance, myelin sheath, axis cylinders, motor end plates, and liposomes in muscle. Ten chameleons were selected also, but at 2-hour

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc., and the Baruch Committee on Physical Medicine.

1 Lewis, W. H., and Richards, A. G., Jr., *Science*, 1945, **102**, 330.

2 Yeager, J. F., and Munson, S. C., *Science*, 1945, **102**, 305.

intervals after the injection of DDT because of the more rapid action of DDT in the chameleon than in the rat. The same microtechnics were employed to study the muscles, peripheral nerves, and spinal cords of the chameleons as in the rat.

The lower extremities in 10 chameleons were quickly skinned and then immersed vertically in Locke's solution, 50°C, for 10 seconds. The muscles of the lower extremities, immediately upon immersion, manifested heat rigor. After immersion, the muscles were quickly excised and prepared for microscopic study. The bloodless muscles of the lower extremities from 10 DDT rats were excised after the muscles were perfused, through the arch of the aorta, with physiological salt solution until clear fluid poured out of an incision in the right auricle of the heart. The muscles were dehydrated *in vacuo* and fed to 10 rats. Seven of the 10 rats manifested DDT toxicity within 3 days. Two hundred fifty mg of dehydrated and triturated muscle, previously perfused, from rats killed with DDT, were suspended in 10 cc of olive oil. Five-tenths of one cc of this suspension were injected intraperitoneally into each of 20 chameleons. Fourteen manifested DDT toxicity within 48 hours and 6 were unaffected. The muscles, peripheral nerves, and spinal cord from 10 normal white rats and 10 chameleons quickly killed by ether were excised, subjected to the microscopic methods of preparation enumerated above, and used as controls. The microscopic observations reported in this paper will be confined dominantly to those demonstrated by the gold technic previously described.[3]

Tests were made *in vitro*, of the chemical reactions of 1% gold chloride with 1% acetylcholine chloride, and of 1% gold chloride with 1% choline chloride. Precipitates of acetylcholine aurichloride and choline aurichloride, respectively, were formed.[4] When this reaction occurred in capillary glass tubes with an inside diameter of 100 μ, evanescent cross striations of periodic precipitation formed, which subsequently disappeared in a uniform diffusion of granules. One percent osmic acid formed a precipitate both with 1% acetylcholine chloride and 1% choline chloride. One percent gold chloride also produced a precipitate with 1% alcoholic solution in each of the following: oleic, palmitic, and stearic acids. There was likewise an interaction between gold chloride and lecithin, and a very strong interaction in the reduction of gold by 1% ascorbic acid and by 1% thiamine hydrochloride.

Results. After the intraperitoneal injection of DDT in the rat, death occurred within 12 to 72 hours, and in the chameleon within 6 to 24 hours. Forty-one of the 50 rats had definite chromodacryorrhea due to overstimulation of the harderian glands which secreted the red-brown porphyrin pigment. This was comparable to the toxic effects of acetylcholine. All the rats had intense salivation, involuntary passage of feces and urine, and clonic, involuntary contractions and tremors of the voluntary muscle, which were the usual physical manifestations of toxicity due to DDT in the rat. In the chameleon the pigmentation of the skin usually changed from green to dark brown, and there were persistent, intermittent contractions of the voluntary muscles. When the chameleons in the terminal stages of DDT toxicity were skinned, there was a continuation of intermittent and incoordinate muscle action that resembled both fibrillation and fasciculation of denervated muscle. This was likewise observed in isolated muscles for 10-20 minutes after excision at 22°C.

The salient microscopic morphologic changes in 0.5% to 15% of the motor innervations in 100 rat and 200 chameleon gastrocnemius muscles after toxicity with DDT (Fig. 2, 3, and 6), in contrast to the normal control muscles (Fig. 1, 4, and 5) in the teased whole muscle fibers and cross sections, were the following: (1) progressive depletion of some epilemmal axons of auriphilic substance and demyelinization of some peripheral nerves; (2) centrifugal discharge from the motor end plate of these auriphilic neurosomes into the myoplasm either as elongated streamers, serial beads, or large elongated masses; (3) the appearance of this auriphilic

[3] Carey, E. J., *Anat. Rec.*, 1941, **81**, 393; *Am. J. Path.*, 1942, **18**, 237.

[4] Loach, J. V., *J. Physiol.*, 1934, **82**, 118.

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FIG. 1 to 3.

Photomicrographs × 200. Normal innervation, Fig. 1, discharge of massive neurosomes from end plates by DDT, Fig. 2 and 3. There is progressive depletion of auriphilic substances in the epilemmal axons, Fig. 2 and 3, contemporaneous with the appearance of the auriphilic neurosomes in the myoplasm. Legend: epa, epilemmal axons; hya, hypolemmal axons; NS, neurosomes. Gold chloride; teased whole gastrocnemius muscle fibers; chameleon.

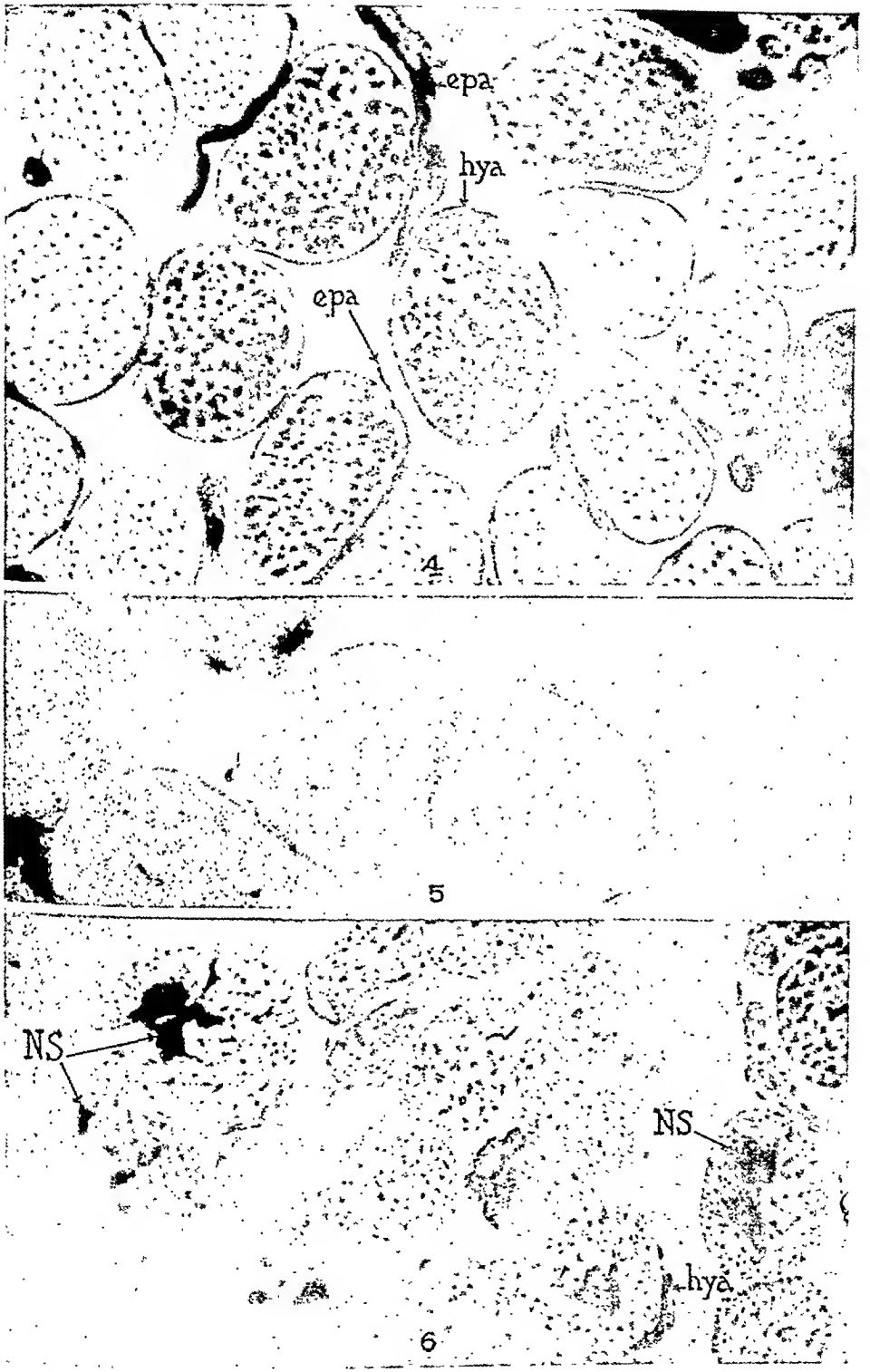

FIG. 4 to 6.

Photomicrographs × 500. Normal coarsely and finely granular muscle fibers, Fig. 4; and agranular fibers, Fig. 5; and agglutination of neurosomes, NS, after DDT, Fig. 6. Legend: epa, epilemmal axons; hya, hypolemmal axons. Gold chloride; cross sections, gastrocnemius muscle, chameleon.

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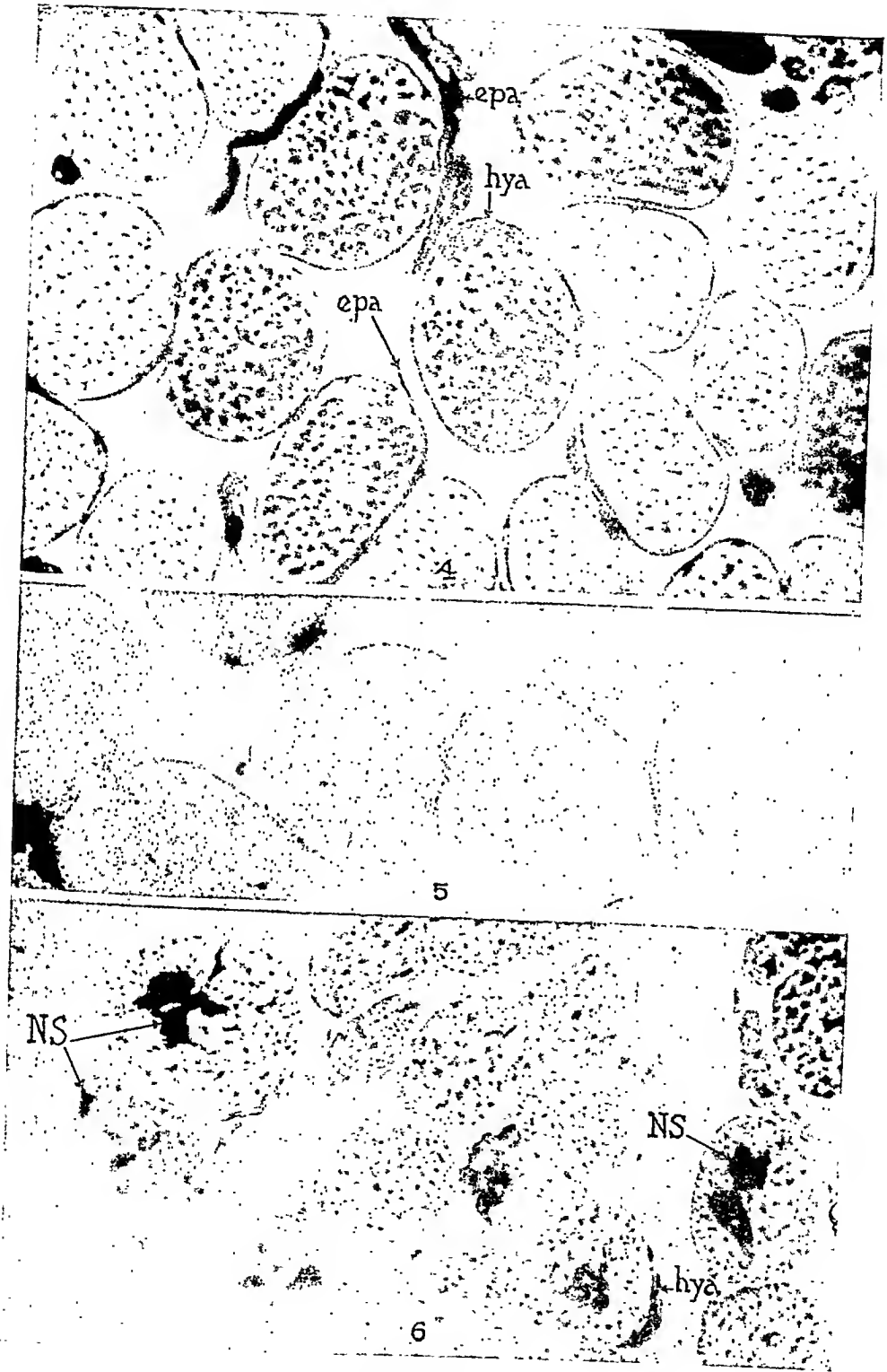

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Photomicrographs × 500. Normal coarsely and finely granular muscle fibers, Fig. 4; and agranular fibers, Fig. 5; and agglutination of neurosomes, NS, after DDT, Fig. 6. Legend: epa, epilemmal axons; hya, hypolemmal axons. Gold chloride; cross sections, gastrocnemius muscle, chameleon.

neurogenic substance in the myoplasm of the muscle fiber by jet-pump action from, and in anatomical continuity with, the motor end plate (Fig. 2 and 3); (4) the subsarcolemmal position of the agglutinated islands of augmented auriphilic substance, seen in whole teased muscle fibers (Fig. 3) and in the cross sections (Fig. 6); (5) an acute atrophy occurring in some muscle fibers affected by DDT (Fig. 6); (6) disappearance of the motor end plates in 10% to 30% of the biopsied gastrocnemius muscles, from 10 chameleons and 10 rats, during the terminal stage of DDT paralysis.

There was an acute atrophy of the muscle fibers in some places. In some muscle fibers dispersion of the granules was lost completely. The granules were agglutinated in large masses in localized spots in some muscle fibers; and this was comparable to the loss of granules in some parts and aggregation in other parts of the specific granular muscle fiber observed 10-18 days after denervation,[5] and after 12 days of total starvation,[6] except water, in the rat. In these denervated gastrocnemius muscles the fine neurosomic granules of Kühne disappeared around the degenerated nerve endings about the 72nd hour after section of the sciatic nerve. There were, however, centrifugal discharges of large, coarse granules into the muscle from the greatly altered nerve terminals, until the distal stump of nerve was depleted of degenerated axonic and myelin substances. This final depletion stage of the distal stump of the sectioned nerve varied in individual rats from 10 to 18 days. The specific structure and dispersal of granules, therefore, in the dark muscle fibers appeared to depend upon the intact nerve endings and normal function of the innervation of muscle.

In the normal muscle fiber of the chameleon there was variation in the distribution of the granular and agranular muscle fibers. The agranular muscle fibers were increased in number by heat rigor of short duration produced by submerging the skinned chameleon in Locke's solution for 10 seconds at 50°C. The agranular fibers produced by this short duration of heat rigor were comparable to the normal agranular fibers (Fig. 5). In heat rigor 90% to 96% of the muscle fibers were agranular, whereas in the normal only 40% to 75% were agranular, 20% to 58% finely granular, and only 2% to 5% coarsely granular. This differential count was based on the observations of 2000 fibers in each of 20 gastrocnemius muscles from 10 chameleons: a total of 40,000 fibers. Although the muscle fibers with greatest diameter were usually pale and agranular, this was not an invariable finding. In the winter animal there was a number of pale fibers with very fine granules, or agranular, that possessed small fiber diameters. Some other medium-sized muscle fibers of the dark type were packed with coarse granules. The normal granules of the muscle fibers varied in diameter from 0.2 to 10 μ.

Some of the auriphilic granules formed elongated bipolar streamers in relation to the muscle nuclei. The granules in the muscle fiber, therefore, were dual in origin: (1) neurosomes discharged from their motor nerve endings and diffused in the myoplasm; and (2) granules discharged from the 2 terminal poles of each of the multiple elongated nuclei in the protoplasm of the muscle. In some places the granules related to the muscle nuclei and the neurosomes continuous with the nerve endings appeared to intermingle and coalesce to form one elongated granular mass. There were many nuclei located in the center of the granular fibers whereas most of the nuclei were located under the sarcolemma in the agranular muscle fibers.

The abnormal auriphilic masses of neurogenic substance produced by DDT were sometimes as long as 450 μ and had in some places (Fig. 3 and 6) a diameter of 50 μ. The normal granules and the abnormal masses of auriphilic neurogenic substance were inconstant in their refractive properties, size, shape, number, and capacity to react to gold, silver, methylene blue, osmic acid, sudan III and IV, sudan black, and alkaline scarlet red. The part of a muscle fiber depleted of its gold staining substance was definitely

5 Carey, E. J., Massopust, L. C., Haushalter, E., Sweeney, J., Saribalis, C., and Raggio, J., *Am. J. Path.*, in press.

6 Carey, E. J., *Anat. Rec.*, 1942, **82**, 403.

agranular when there was an aggregation of this substance produced by DDT in another part of the same fiber. The massive aggregation of auriphilic substance in the myoplasm of the voluntary muscle fiber was in anatomic continuity in many instances with the auriphilic substance of the hypolemmal and epilemmal axons undergoing depletion by the action of DDT. That at least some of the auriphilic masses were neurogenic in origin is proved by the following microscopic evidence: (1) there is frequently a continuous anatomic relationship between this auriphilic substance in the muscle and the nerve ending; (2) there is a similarity of reaction of this substance in nerve and muscle with gold; (3) there is an excessive and massive accumulation of this auriphilic substance in muscle parallel with the exhaustion of the nerve supply of its auriphilic substance.

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[15] Knoll, P., *Z. f. Heilk.*, Berlin, 1880-81, **1**, S. 255; *Denkschr. d. k. Akad. d. Wissensch. Wien. Math. Naturw. Kl.*, Wien, 1891, **58**.

[16] Kölliker, A., *Z. f. Wissensch. Zool.*, Leipzig, 1857, **8**, 311; *ibid.*, 1888, **47**, S. 689.

[17] Albrecht, E., *Deutsche. path. Gesellsch.*, 1902, **5**, S. 7; *ibid.*, 1903, **6**, S. 63; *Ergeb. der allg. Path. u. Path. Anat.*, 1907, **11**, Abth. 2, S. 1166.

[18] Bell, E. T., *Anat. Rec.*, 1910, **4**, 199; *Internat. Monatsch. f. Anat. u. Physiol.*, 1911, **28**, S. 297; *J. Path. and Bact.*, 1912, **17**, 147.

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In the normal muscle fiber of the chameleon there was variation in the distribution of the granular and agranular muscle fibers. The agranular muscle fibers were increased in number by heat rigor of short duration produced by submerging the skinned chameleon in Locke's solution for 10 seconds at 50°C. The agranular fibers produced by this short duration of heat rigor were comparable to the normal agranular fibers (Fig. 5). In heat rigor 90% to 96% of the muscle fibers were agranular, whereas in the normal only 40% to 75% were agranular, 20% to 58% finely granular, and only 2% to 5% coarsely granular. This differential count was based on the observations of 2000 fibers in each of 20 gastrocnemius muscles from 10 chameleons: a total of 40,000 fibers. Although the muscle fibers with greatest diameter were usually pale and agranular, this was not an invariable finding. In the winter animal there was a number of pale fibers with very fine granules, or agranular, that possessed small fiber diameters. Some other medium-sized muscle fibers of the dark type were packed with coarse granules. The normal granules of the muscle fibers varied in diameter from 0.2 to 10 μ.

Some of the auriphilic granules formed elongated bipolar streamers in relation to the muscle nuclei. The granules in the muscle fiber, therefore, were dual in origin: (1) neurosomes discharged from their motor nerve endings and diffused in the myoplasm; and (2) granules discharged from the 2 terminal poles of each of the multiple elongated nuclei in the protoplasm of the muscle. In some places the granules related to the muscle nuclei and the neurosomes continuous with the nerve endings appeared to intermingle and coalesce to form one elongated granular mass. There were many nuclei located in the center of the granular fibers whereas most of the nuclei were located under the sarcolemma in the agranular muscle fibers.

The abnormal auriphilic masses of neurogenic substance produced by DDT were sometimes as long as 450 μ and had in some places (Fig. 3 and 6) a diameter of 50 μ. The normal granules and the abnormal masses of auriphilic neurogenic substance were inconstant in their refractive properties, size, shape, number, and capacity to react to gold, silver, methylene blue, osmic acid, sudan III and IV, sudan black, and alkaline scarlet red. The part of a muscle fiber depleted of its gold staining substance was definitely

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of the intramuscular medullated nerves.

The augmentation and aggregation of neurogenic substances in the voluntary muscle fibers affected by DDT parallels the abnormal response and subsequent paralysis through axonic exhaustion by chemical denervation of some muscle fibers. It is suggested that the normal dark and granular muscle fiber be designated as the neurosomic muscle fiber and that the light and agranular fiber be called the aneurosomic muscle fiber. This terminology is based on the hypothesis that there is a periodic discharge and disappearance of the neurogenic granules in the fibrillar protoplasm of the same voluntary muscle fiber observed at different periods of time, and the hypothesis itself is supported by the suggestive experimental evidence of the effects of DDT.

The observations reported in this paper are, in general, consistent with the views expressed, over one hundred years ago, by Doyère.[23] He observed the junction between the living nerve and muscle in the microscopic Tardigrade (Spallanzani) also called the "water bear." Its body is a transparent muscular bag. Doyère stated, page 346, that "the relation of the terminal nerve filaments with the muscles cannot be distinguished," and in Fig. 4, plate 17, he illustrates the junction of the granular nerve fiber and the granular muscle fiber as morphologically continuous.

Summary. The limited experimental evidence presented in this paper tends to support the statement that DDT increases the discharge of auriphilic neurogenic substances from some motor end plates into the myoplasm of some of the voluntary muscle fibers. These neurogenic substances form massive aggregates of neurosomes in parts of some voluntary muscle fibers. This results in a partial or complete dissociation of the neurogenic from the myogenic substances in the muscle fiber. Auriphilic granules are likewise found at the 2 poles of the elongated nuclei in the dark muscle fibers. The supporting evidence that some of the auriphilic granules and masses found after DDT toxicity are neurogenic in origin follows: (1) anatomical continuity of the auriphilic bodies in muscle with the auriphilic hypolemmal axons of the motor end plates; (2) similarity of staining reaction of the masses in muscle and axons of nerve endings with gold impregnation; (3) massive aggregation of auriphilic bodies in some muscle fibers contemporaneous with the centrifugal depletion of the related nerve axons and endings of their auriphilic substances. Presumptive evidence is presented that the normal fiber types in voluntary muscle are dependent, in part, upon the periodic alternation of the discharge and disappearance, by chemical action, of the neurogenic granules in the same muscle fiber at different time periods. The limited evidence also indicates that DDT poisoning may be produced in normal rats by feeding, and in chameleons by the intraperitoneal injection of the previously perfused, dehydrated, and emulsified muscles of rats manifesting toxicity with DDT.

[23] Doyère, M., *Annales d. Sciences Naturelles*, second series, 1840, **14**, 269.

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Diffusion of Sulfonamides and Penicillin into Fibrin.*

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Until the advent of penicillin, subacute bacterial endocarditis largely resisted all forms of therapy. The persistence of the disease is due to the survival of the bacteria in the valve locus. The organisms in the vegetations are separated from the blood stream by a layer of fibrin and it has been

* This study was carried out with the support of the Dorothy H. and Lewis Rosenstiel Foundation.

It is suggested that the locations of the muscle nuclei are related to the mechanical effects of the chemical reactions produced by the discharge, hydrolysis, and disappearance of the granular neurosomes projected into the myoplasm from the nerve endings. It is likewise suggested that the fiber types in muscle appeared not to be fixed histologic structures but that they readily are transformed one into the other, dependent upon the phase of the metabolic cycle of nerve and muscle in which the tissue ceased activity by the impregnation with gold. The periodic changes in the histochemistry of muscle appeared to determine the granular and the agranular types of muscle fibers independently of the gross color of the muscle. The observations reported in the chameleon in this paper are made upon the grossly pale reptilian muscle, and the granular fibers have no relation to the so-called "red" muscle.

The same axis cylinder may divide and end in 2 motor end plates, one retracted in the granular muscle fiber and the other expanded in the agranular muscle fiber. This is suggestive evidence that the same axis-cylinder of a nerve, upon division, may terminate in muscle fibers fixed in different phases of either the discharge or hydrolysis of neurosomes in the metabolic cycle of nerve and muscle. The greater number of granular fibers found in the slower than in the faster reacting muscle is assumed to be related to the differential speed of metabolism and response to the chemical irritation produced by gold impregnation. If this assumption is true, then a greater number of granular fibers would be found in the slower than in the faster reacting muscles. These granular fibers, therefore, are not related to hemoglobin pigmentation and the adjective "red" should be discarded.

Denny-Brown[11] cut the ventral roots of the sciatic nerve in the cat. Two weeks later he found that the distribution and depth of staining of dark and light fibers in the gastrocnemius and soleus had remained the same on the operated side as on the control side. We have duplicated this experiment in 50 rats[6] and 10 cats. The definitive dark fibers disappear in from 10 to 18 days in the rat, and from 21 to 36 days in the cat. There is no way, based upon the use of either fat stains of alkaline sudan III, scarlet red, osmic acid or metallic impregnation with gold or silver, of clearly differentiating from one another acetylcholine, choline, lecithin, and other products of the breakdown of complex lipoids. Seventy-two hours after nerve section in the rat, acetylcholine is not identified by Dale, Feldberg, and Vogt,[22] yet there is a periodic discharge of some complex lipoids, in the form of very coarse granules, from the degenerated end plate, and these granules react to osmic acid, alkaline sudan III, and to gold chloride. This centrifugal discharge of the degenerated axonic and myelin material of the distal stump of the cut sciatic nerve into the denervated muscle continues until complete substantial exhaustion occurs. It is because of this fact, and species variation in degeneration of nerves, that Denny-Brown[11] did not observe any great change in the disappearance of the muscle granules in the narrow, dark fibers 14 days after nerve section in the cat. The dark, granular muscle fiber disappears when a sufficient length of time has elapsed after nerve section as well as after starvation. Denervation[6] results in a material, lipoidal exhaustion of the distal stump of the sciatic nerve, whereas starvation[6] produces either a partial or complete block in the substantial transmission of neurogenic substances in some fibers of the muscle. This is evident by the great enlargement of some of the nerve axons extrinsic to the sarcolemma[6] and the retraction of the hypolemmal axons and disappearance of the granules of Kühne.

The effect of DDT on the neuromuscular apparatus results in an augmented discharge and aggregation of neurosomes in the myoplasm. DDT produces dissociation of the neurogenic and myogenic substances in the voluntary muscle fiber. DDT likewise finally produces a chemical denervation of some muscle fibers microscopically detected by the disappearance of some of the motor end plates. DDT also causes demyelinization of some

[22] Dale, H. H., Feldberg, W., and Vogt, M., *J. Physiol.*, 1936, **86**, 353.

TABLE I.
Inhibition Zones Diameter in mm.
Diameter of zones of inhibition in mm on agar and fibrin plates produced by sodium sulfathiazole, sodium sulfadiazine, and disodium phosphate.

	M/25 Sodium-sulfathiazole		M/25 Sodium-sulfadiazine		M/25 Disodium-phosphate	
hr	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin
24	0	0	0	0	0	0
48	12	0	0	0	0	0
72	15	0	14	0	0	0
96	18	0	18	0	0	0
120	18	0	18	0	0	0

TABLE II.
Diameter of Zones of Inhibition in mm.

Penicillin units/ml	0.5		1.0		2.0		4.0	
hr	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin
24	9	9	9	9	11	9	11	10
48	10	9	11	9	12	9.5	15	12
72	11	9	12	10	12	10	18	12
96	12	10	13	12	14	13	18	14
120	14	10	16	12	16	14		
144	14	10	16	12	16	14		
168	14	10	16	12	16	14		

sodium sulfadiazine and the third with the control M/25 disodium phosphate. Readings were made at 24-hour intervals and the averages of the 3 readings taken. Table I shows the results of these experiments. It is evident that sodium sulfathiazole and sodium sulfadiazine diffuse into agar but show no reaction in fibrin. The control solution shows no inhibition in either the agar or fibrin plates indicating that the alkalinity of the sodium sulfonamides is not a factor in the reactions.

Penicillin. A number of experiments were carried out with varying concentrations of penicillin. A zone of inhibition appeared in both the agar and fibrin plates in 24 hours. Although the inhibition zones were somewhat larger in the agar as compared with fibrin plates, the difference was relatively slight, indicating a comparatively free diffusion of penicillin into fibrin. Table II shows the results of a typical experiment.

Discussion. These results indicate that penicillin diffuses freely into fibrin as compared with sulfonamide compounds. The conclusion seems justifiable that the greater effectiveness of penicillin in subacute bacterial endocarditis is at least in part due to this superior diffusibility. The fact that there is a relationship between the chemotherapeutic activity of a compound and its ability to permeate fibrin supports the concept of a fibrin barrier as the mechanism which permits the persistence of the infection on the valve. It is clear that in evaluating the efficiency of a drug in the treatment of bacterial endocarditis, the ability to penetrate fibrin requires important consideration.

A further implication of the present study relates to the use of anticoagulants in the treatment of subacute bacterial endocarditis. With the purpose of lessening the deposition of fibrin on the diseased valve, the administration of heparin has been used as an adjunct to antibacterial agents. Although some clinical reports suggest that heparin is beneficial, others indicate that the results of penicillin therapy are not improved by its use. The present experiments would tend to minimize the importance of heparin as it is shown that fibrin has little retarding effect on the activity of penicillin.

Summary and Conclusion. Using a modification of the cup assay method on agar and fibrin plates, there is no evidence of penetration of sulfathiazole and sulfadiazine into

shown that antibacterial agents penetrate fibrin poorly or not at all. Friedman[1] studied the effect of 3 germicides, gentian violet, merthiolate and Vuzino-toxine camphorated on the *Streptococcus viridans* growing imbedded in a fibrin mass. He compared the effect with that occurring in ordinary broth cultures of the same organism and found that the fibrin mass had a striking retarding effect on the bactericidal action of these compounds. Friedman[2] also demonstrated the inability of sulfanilamide and sulfapyridine to eradicate a focus of streptococci growing in and protected by a fibrin-platelet mass. Duncan and Faulkner[3] were unable to demonstrate any appreciable penetration of sulfonamide compounds into blood clots in periods of 24 hours to 15 days. These observations suggest that failure of therapy in bacterial endocarditis may be due to an ineffective contact between chemotherapeutic agents and the bacteria on the valves as a result of impermeability of the fibrin barrier.

The sulfonamide compounds[4] and penicillin[5] inhibit the growth of *Streptococcus viridans* in the test tube. However, in bacterial endocarditis with the organisms lodged in the heart valve, penicillin has shown a striking therapeutic effect while the sulfonamides have at best exhibited only an occasional and usually temporary beneficial action. The present study is an attempt to determine whether the greater effectiveness of penicillin can be ascribed to a superior diffusion of this substance into fibrin. A comparison was made of the diffusibility into fibrin of the relatively ineffective sulfonamide compounds with that of penicillin.

Procedure. A modification of the cup assay method for penicillin was utilized. Agar plates were prepared using *Bacillus subtilis* as the test organism following the technic of Foster and Woodruff.[6] Fibrin plates were prepared in the following manner. Dried fraction I of human plasma† was reconstituted by the addition of saline, containing 10% glucose. Twenty ml portions were poured into petri dishes and 0.2 ml of a spore suspension of *B. subtilis* added to each plate.

The plates were shaken to permit an even distribution of the bacterial suspension. To each plate 0.2 ml of a solution of bovine thrombin was added. The thrombin was prepared by dissolving 5000 units in 5 ml of isotonic saline. The fibrin hardens within 2 minutes after the addition of the thrombin. Three penicillin assay cups were placed equidistantly on each agar and fibrin plate. The 3 cups were filled with the substance to be tested, permitting the reading of the results in triplicate. The plates were kept at room temperature as preliminary experiments showed that liquefaction occurred in the fibrin at incubator temperature. Measurements of the diameter of the zones of inhibition were made at 24-hour intervals.

Sulfonamide Compounds. Sulfathiazole and sulfadiazine were used in this study. Relatively dilute solutions of these compounds did not produce a definite inhibition in either the agar or fibrin plates. More concentrated solutions (M/25) of the sodium salts of these compounds showed definite zones of inhibition in the agar but there was no evidence of inhibition in the fibrin. To assay the possible effect of the alkalinity of these solutions, a control of disodium phosphate in M/25 solution, adjusted to the same pH as that of the sodium sulfonamides was utilized. One agar and one fibrin plate were set up with the 3 cups filled with the M/25 sodium sulfathiazole, another pair with M/25

1 Friedman, M., *J. Pharmacol. and Exp. Therap.*, 1938, **63**, 173.

2 Friedman, M., *Arch. Int. Med.*, 1941, **67**, 921.

3 Duncan, C. N., and Faulkner, J. M., *Am. J. Med. Sc.*, 1940, **200**, 492.

4 Orgain, E. S., and Poston, M. A., *Arch. Int. Med.*, 1942, **70**, 777.

5 Dawson, M. H., Hobby, G. L., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 101.

6 Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1944, **47**, 43.

† This plasma fraction was prepared under a contract between the Office of Scientific Research and Development, and Harvard University, from blood collected by the American Red Cross. This was supplied by the Department of Physical Chemistry, Harvard Medical School. In this fraction, 60% of the protein is fibrinogen and the remainder is albumin and globulin.

TABLE I.

Inhibition Zones Diameter in mm.

Diameter of zones of inhibition in mm on agar and fibrin plates produced by sodium sulfathiazole, sodium sulfadiazine, and disodium phosphate.

	M/25 Sodium-sulfathiazole		M/25 Sodium-sulfadiazine		M/25 Disodium-phosphate	
hr	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin
24	0	0	0	0	0	0
48	12	0	0	0	0	0
72	13	0	14	0	0	0
96	18	0	18	0	0	0
120	18	0	18	0	0	0

TABLE II.

Diameter of Zones of Inhibition in mm.

Penicillin units/cc	0.5		1.0		2.0		4.0	
hr	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin	Agar	Fibrin
24	9	9	9	9	11	9	11	10
48	10	9	11	9	12	9.5	13	12
72	11	9	12	10	12	10	15	12
96	12	10	13	12	14	13	15	14
120	14	10	16	12	16	14		
144	14	10	16	12	16	14		
168	14	10	16	12	16	14		

sodium sulfadiazine and the third with the control M/25 disodium phosphate. Readings were made at 24-hour intervals and the averages of the 3 readings taken. Table I shows the results of these experiments. It is evident that sodium sulfathiazole and sodium sulfadiazine diffuse into agar but show no reaction in fibrin. The control solution shows no inhibition in either the agar or fibrin plates indicating that the alkalinity of the sodium sulfonamides is not a factor in the reactions.

Penicillin. A number of experiments were carried out with varying concentrations of penicillin. A zone of inhibition appeared in both the agar and fibrin plates in 24 hours. Although the inhibition zones were somewhat larger in the agar as compared with fibrin plates, the difference was relatively slight, indicating a comparatively free diffusion of penicillin into fibrin. Table II shows the results of a typical experiment.

Discussion. These results indicate that penicillin diffuses freely into fibrin as compared with sulfonamide compounds. The conclusion seems justifiable that the greater effectiveness of penicillin in subacute bacterial endocarditis is at least in part due to this superior diffusibility. The fact that there is a relationship between the chemotherapeutic activity of a compound and its ability to permeate fibrin supports the concept of a fibrin barrier as the mechanism which permits the persistence of the infection on the valve. It is clear that in evaluating the efficiency of a drug in the treatment of bacterial endocarditis, the ability to penetrate fibrin requires important consideration.

A further implication of the present study relates to the use of anticoagulants in the treatment of subacute bacterial endocarditis. With the purpose of lessening the deposition of fibrin on the diseased valve, the administration of heparin has been used as an adjunct to antibacterial agents. Although some clinical reports suggest that heparin is beneficial, others indicate that the results of penicillin therapy are not improved by its use. The present experiments would tend to minimize the importance of heparin as it is shown that fibrin has little retarding effect on the activity of penicillin.

Summary and Conclusion. Using a modification of the cup assay method on agar and fibrin plates, there is no evidence of penetration of sulfathiazole and sulfadiazine into

fibrin. Penicillin diffuses almost as well into fibrin as into agar. It is suggested that the diffusibility of penicillin into fibrin is an important factor in the efficacy of this substance in the treatment of subacute bacterial endocarditis.

We are indebted to Dr. Gordon Alles for helpful suggestions.

15381

Serum Levels After Repository Injections of Penicillin.

EDWIN M. ORY, CLARE WILCOX, AND MAXWELL FINLAND.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

There is considerable interest in the development of methods for administering penicillin which are both simple and efficient. Oral administration would be the obvious solution were it not for the marked irregularity of absorption as well as the low efficiency of this method with the materials thus far available.[1-4] Various attempts have been made to maintain and prolong the effective concentrations of penicillin after parenteral administration with the hope of reducing the number of injections necessary. Most promising at the present time is the use of a repository injection of a relatively large dose in a medium from which absorption takes place slowly. Among the various combinations proposed, the mixtures of beeswax in peanut oil have been studied most extensively.[5-9] With this method effective serum levels can be maintained quite regularly over a 12-hour period and in most instances for 18 to 24 hours, but the concentrations after more than 12 hours following a dose of 300,000 units or less may be quite irregular or not measurable. While the beeswax-peanut oil mixtures have been used successfully in several clinics, their high melting point and the marked viscosity of the preparations make the injections of these materials technically difficult for most inexperienced persons. Substances which are more easily manipulated at ordinary room temperatures would be much more desirable.

Freund and Thomson[10] proposed the use of a simple, rapid technic of preparing water-in-oil emulsions of penicillin. The penicillin is put into solution in a small volume of saline and added to a mixture of a lanolin-like substance (Falba) and peanut oil and thoroughly emulsified before the injection. This method has been used by Cohn *et al.*[11] in the treatment of 52 cases of acute gonorrhea by the single injection of the emulsion containing 150,000 units of penicillin, with only 2 failures. The serum levels obtained in 3 subjects given the penicillin in this manner were somewhat better sustained than in 2 others in which the same amount of penicillin was given in saline. Similar or identical materials have been prepared commercially (Solvecillin, Pendil, Emulgen, etc.) and have the advantage that they flow readily at or

1 Free, A. H., Parker, R. F., and Biro, B. E., *Science*, 1945, **102**, 666.

2 Cutting, W. C., *et al.*, *J. A. M. A.*, 1945, **129**, 425.

3 Finland, M., Meads, M., and Ory, E. M., *J. A. M. A.*, 1945, **129**, 315.

4 McDermott, W., *et al.*, *Science*, 1946, **103**, 359.

5 Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

6 Romansky, M. J., and Murphy, R. J., *J. A. M. A.*, 1945, **128**, 404.

7 Romansky, M. J., and Rittman, G. E., *New England J. Med.*, 1945, **233**, 577.

8 Leifer, W., Martin, S. P., and Kirby, W. M. M., *New England J. Med.*, 1945, **233**, 583.

9 Kirby, W. M. M., *et al.*, *J. A. M. A.*, 1945, **129**, 940.

10 Freund, J., and Thomson, K. J., *Science*, 1945, **101**, 468.

11 Cohn, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 145.

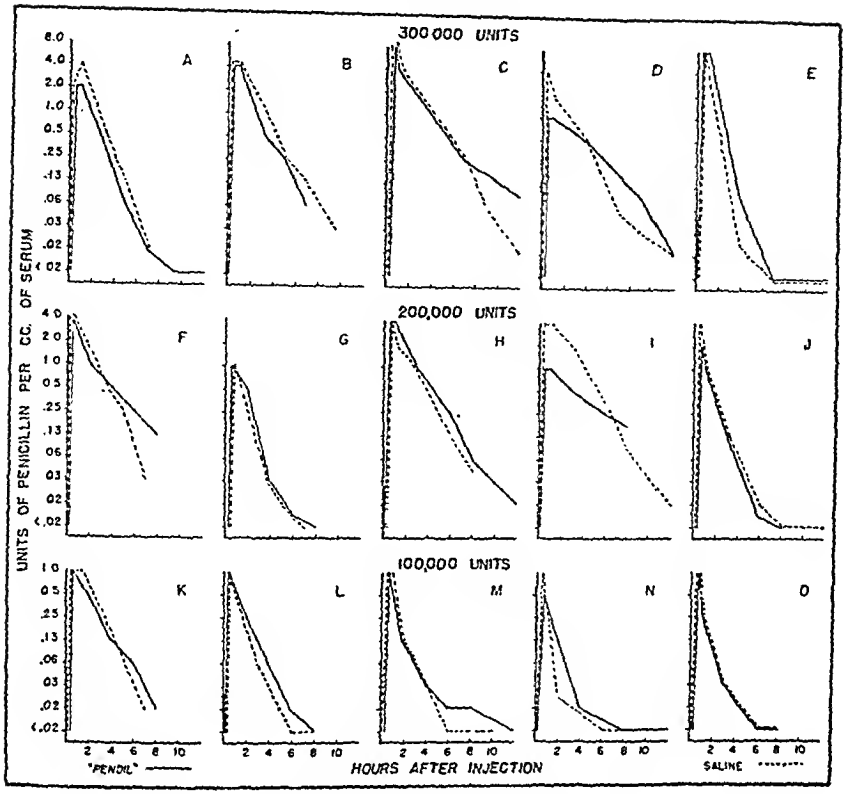

FIG. 1.

Serum penicillin concentrations in the same subjects after intramuscular injections of various doses in saline and in a water-in-oil emulsion.

slightly above room temperature and can therefore be easily manipulated. From that point of view, this type of mixture would be much preferable to the thicker beeswax mixtures, which readily solidify at the same temperatures.

In view of the fact that there may be considerable individual variations in the absorption of penicillin even after intramuscular injections of the same dose, it seemed essential to compare serum levels obtained from any given dose in the repository injection with those obtained with saline solutions in the same individuals.

Intramuscular doses of 100,000, 200,000 and 300,000 units, each in a volume of 4.5 cc were used. The subjects were ward patients who had not recently received any chemo- or antibiotic therapy. About one-half of the subjects were given a single dose of penicillin in saline and 3 or more days later the same amount of penicillin was given in water-in-oil emulsion* as advocated by Freund and Thomson.[10] The other subjects received the doses in the reverse order. Blood was drawn at intervals after the injection and the concentration of penicillin in the serum determined by a slight modification of the serial dilution method of Rammelkamp.[12] The minimum concentration detectable by this method was 0.0156 unit per cc of serum.

The results are shown in Fig. 1. There were slight variations in the maximum concentrations obtained and somewhat greater individual variations in the maintenance of serum levels after the same dose. The larger doses gave higher and more prolonged levels in either menstruum. There was no constant

* "Pendil," generously supplied by Endo Products, Inc., through the courtesy of Dr. Samuel M. Gordon.

[12] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, 51, 95.

fibrin. Penicillin diffuses almost as well into fibrin as into agar. It is suggested that the diffusibility of penicillin into fibrin is an important factor in the efficacy of this substance in the treatment of subacute bacterial endocarditis.

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15381

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4 McDermott, W., *et al.*, *Science*, 1946, **103**, 359.

5 Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

6 Romansky, M. J., and Murphy, R. J., *J. A. M. A.*, 1945, **128**, 404.

7 Romansky, M. J., and Rittman, G. E., *New England J. Med.*, 1945, **233**, 577.

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9 Kirby, W. M. M., *et al.*, *J. A. M. A.*, 1945, **129**, 940.

10 Freund, J., and Thomson, K. J., *Science*, 1945, **101**, 468.

11 Cohn, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 145.

TABLE I.
Effect of Pyridoxin Deficiency upon Serum Antibodies.

Group	Experiment	No. of rats	Average Initial body wt, g	Average Final body wt, g	Average Thymus wt, g	Agglutinin titers Avg	Agglutinin titers Range	Hemolysin titers Avg	Hemolysin titers Range
I	Pyridoxin deficient	9	47	83	.047	1:0.4	0-1:4	1:13	0-1:80
II	Paired weighed	7	46	78	.182	1:64	1:32-1:128	1:412	1:160-1:640
III	Control	8	45	160	.395	1:36	1:10-1:80	1:68	1:20-1:160

on a diet deficient in pyridoxin but otherwise adequate.[4] Daily weights were recorded in these animals. Restricted amounts of the complete diet were fed to the second group in order to duplicate the growth retardation produced by pyridoxin deficiency (paired weighing). The third group received the complete diet *ad libitum*. During the fifth week of the dietary experiment all animals were immunized against washed sheep erythrocytes. Three intraperitoneal injections of 0.5 ml of a 5% suspension of red cells were administered on alternate days. Five days after the last injection the animals were exsanguinated under sterile precautions. Hemagglutinin and hemolysin determinations were carried out on individual sera by the usual method of serum dilution in 2-fold steps. The titer endpoints adopted were 3 plus for agglutinins and 2 plus for hemolysins.

Immediately after the bleeding the thymus and other organs were weighed and prepared for histological study.

Results. The data are summarized in Table I. It is apparent that there occurred growth retardation in Groups I and II, with very low quantities of circulating antibodies in the pyridoxin-deficient animals. Although the scatter of the antibody values is great in both control groups, the difference between these and the deficient group is most striking. Of the 9 pyridoxin-deficient animals 6 showed no measureable agglutinins or hemolysins and 3 had only very low titers. In the 2 control groups all 16 animals had measurable antibody titers, and 13 of these were comparatively high.

The extreme degree of lymphoid atrophy was apparent not only from the weight of the thymus, but also from their morphology. In the thymi and lymph nodes of the deficient animals there was observed a pronounced reduction in the number of lymphocytes.

Discussion. From the data presented it is apparent that animals fed a diet deficient in pyridoxin and then immunized exhibited little or no circulating antibodies. The immunization was begun at a time when lymphoid atrophy was advanced. From the data on hand, however, it cannot be decided that the suppression of the serum antibodies was a consequence of the lymphoid atrophy: It is possible that both alterations occur independently in pyridoxin deficiency. Further study of this point is now in progress.

Summary. Male albino rats immunized in a state of pyridoxin deficiency developed antibody levels in the serum far below those of inanition controls (paired weighed) and full controls.

We wish to express our thanks to Dr. M. Mayer for giving generously of his time and advice on the immunological procedure.

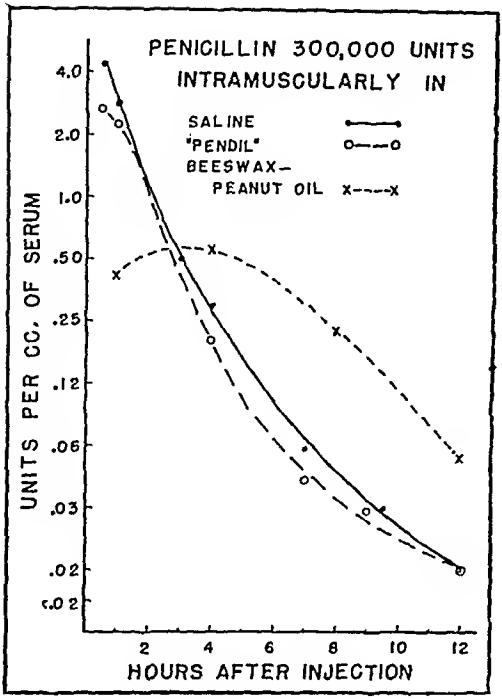


FIG. 2.
Serum penicillin concentrations after intramuscular injections of 300,000 units of penicillin in 3 vehicles.

or striking difference between the results obtained in the same subject after the same dose given in the 2 vehicles.

In Fig. 2 are shown the mean concentrations obtained over a 12-hour period after intramuscular injections of 300,000 units of penicillin given in saline, in Pendil, and in 4.8% beeswax in peanut oil (Delacillin, Squibb), the latter being contained in a volume of 1 cc. Each curve is based upon the results obtained in 6 subjects. It is seen that the maximum concentration obtained with the beeswax-peanut oil mixture was appreciably lower, but the levels were much better sustained than with the same dose in saline or in Pendil.

Conclusions. When tested in the same subject, the serum concentrations obtained after a single injection of penicillin in a water-in-oil emulsion were not superior to those obtained with the same dose given in the same volume of saline, and the levels were not better sustained. A dose of 300,000 units in 1 cc of 4.8% beeswax in peanut oil gave lower maximum concentrations, but the serum levels were better sustained over a 12-hour period.

15382 P

Suppression of Circulating Antibodies in Pyridoxin Deficiency.

HERBERT C. STOERK AND HERMAN N. EISEN.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

There has recently accumulated evidence to indicate that there may be an important relationship between lymphocytes and antibodies.[1,2,3] Data suggesting that pyridoxin may be a factor essential for the maintenance of lymphoid tissue have been reported previously.[4,5,6] It therefore seemed of interest to study the effect of pyridoxin deficiency on circulating antibodies.

Methods. Twenty-four male albino rats of the Sherman strain, close to 4 weeks of age, were divided into 3 groups of litter mates.

The animals in the first group were weaned

1 Ehrich, W. F., and Harris, T. N., *Science*, 1945, **101**, 28.

2 Daugherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 295.

3 Daugherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 135.

4 Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 151.

5 Stoerk, H. C., *Fed. Proc.*, 1946, **5**, 227.

6 Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., in press.

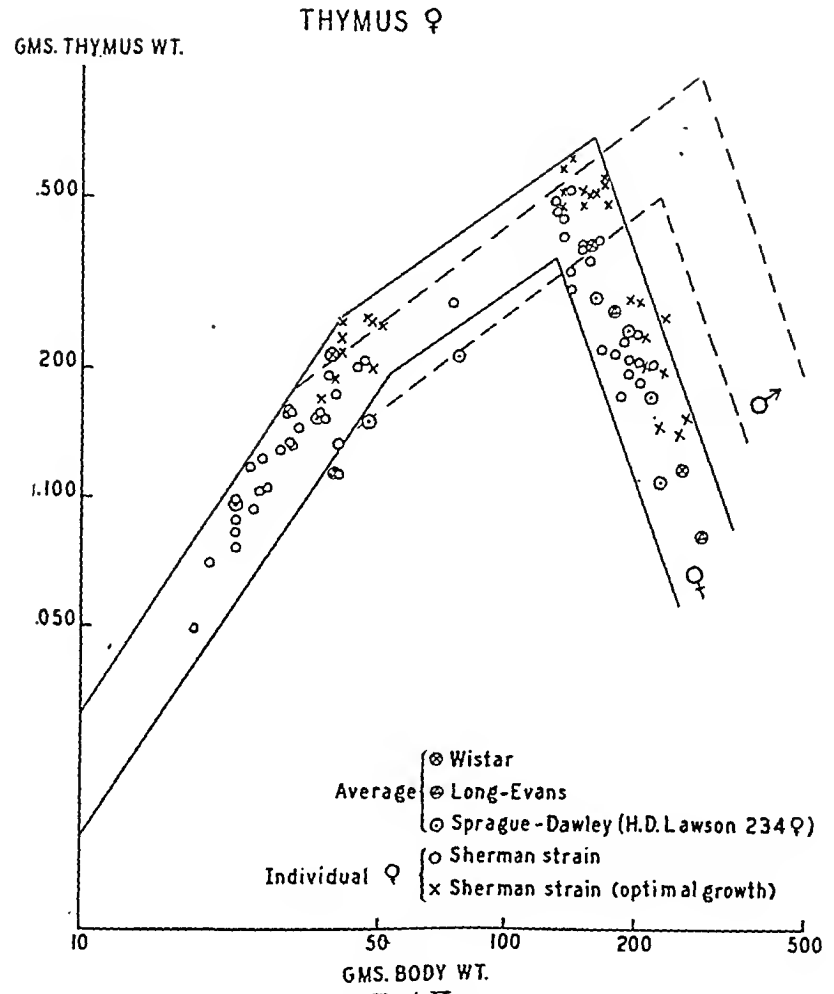


Chart II.

Log./Log. plot of thymus weight on body weight in female albino rats.

ditions probably close to optimal.

2. To study the extent of thymic atrophy (accidental involution) produced by closely controlled experimental conditions.

All of the experimental conditions induced, represented adverse bodily conditions and were associated with growth, retardation or body weight loss. The extent of the harmful stimulus was judged by its effect on body weight.

In all conditions where effects on thymic tissue were observed the lymphoid tissue appeared similarly affected. This was observed from the histology of lymph nodes and spleen.

Several attempts have been made in the past to determine the norm for thymus size in the rat over a range of age or body weight.[1-6] Only part of the data, however, were collected recently enough to take into account the newer knowledge of the dietary requirements in this species.

[1] Donaldson, H. H., *The Rat*, 1924, Mem. Wistar Inst.

[2] Jackson, C. M., *Anat. Rec.*, 1937, **68**, 371.

[3] Watanabe, T., *Trans. Jap. Path. Soc.*, 1929, **17**, 332.

[4] Moment, G. B., *J. Exp. Zool.*, 1933, **65**, 359.

[5] Lawson, H. D., *et al.*, *Endocrinology*, 1942, **31**, 129.

[6] Stoerk, H. C., *Endocrinology*, 1944, **34**, 329.

15383

Effects of Calcium Deficiency and Pyridoxin Deficiency on Thymic Atrophy (Accidental Involution).

HERBERT C. STOERK.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

In a variety of experimental conditions atrophic thymus glands have been described by many observers. In many of the cases this atrophy has been attributed to the variable introduced by the experiment. The specificity of the observed effect, however, was not ascertained by sufficient controls.

In the following it has been attempted:

1. To establish the approximate norm for thymus size in albino rats raised under con-

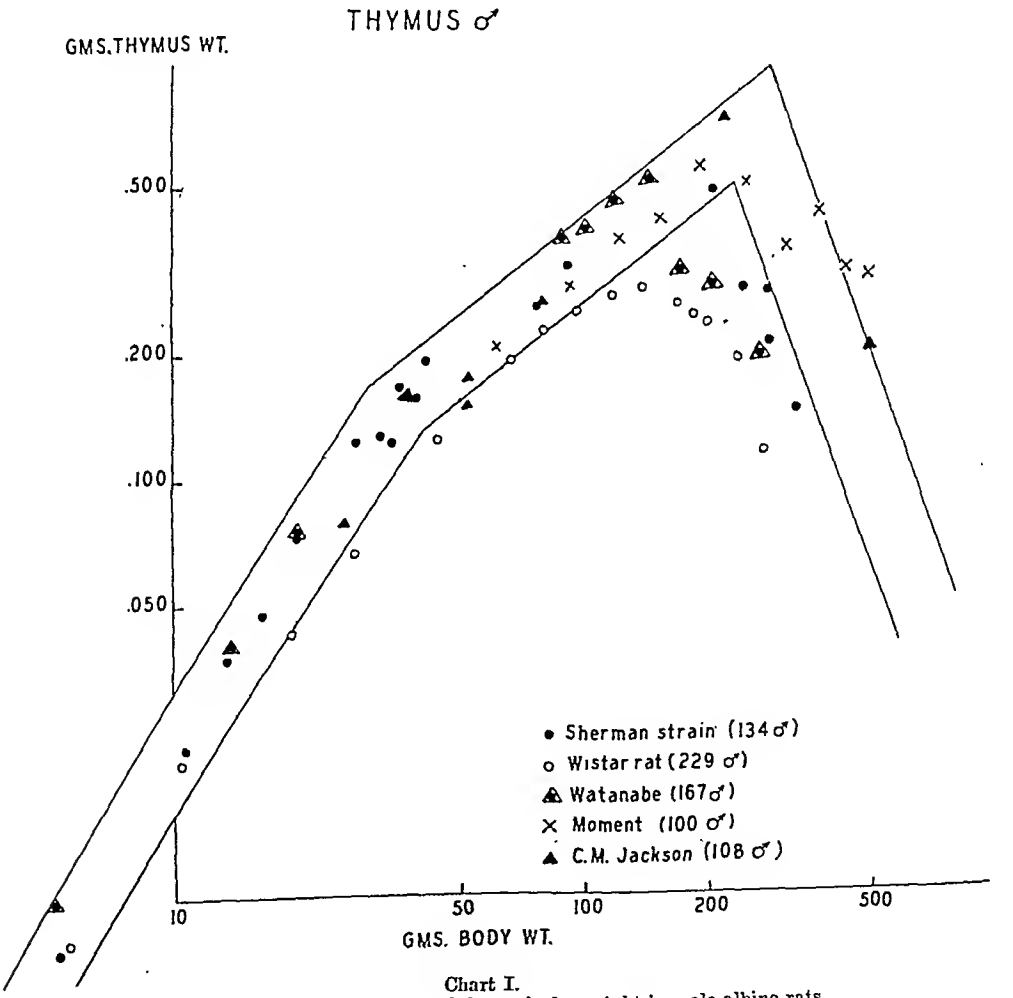


Chart I.
Log./Log. plot of thymus weight on body weight in male albino rats.

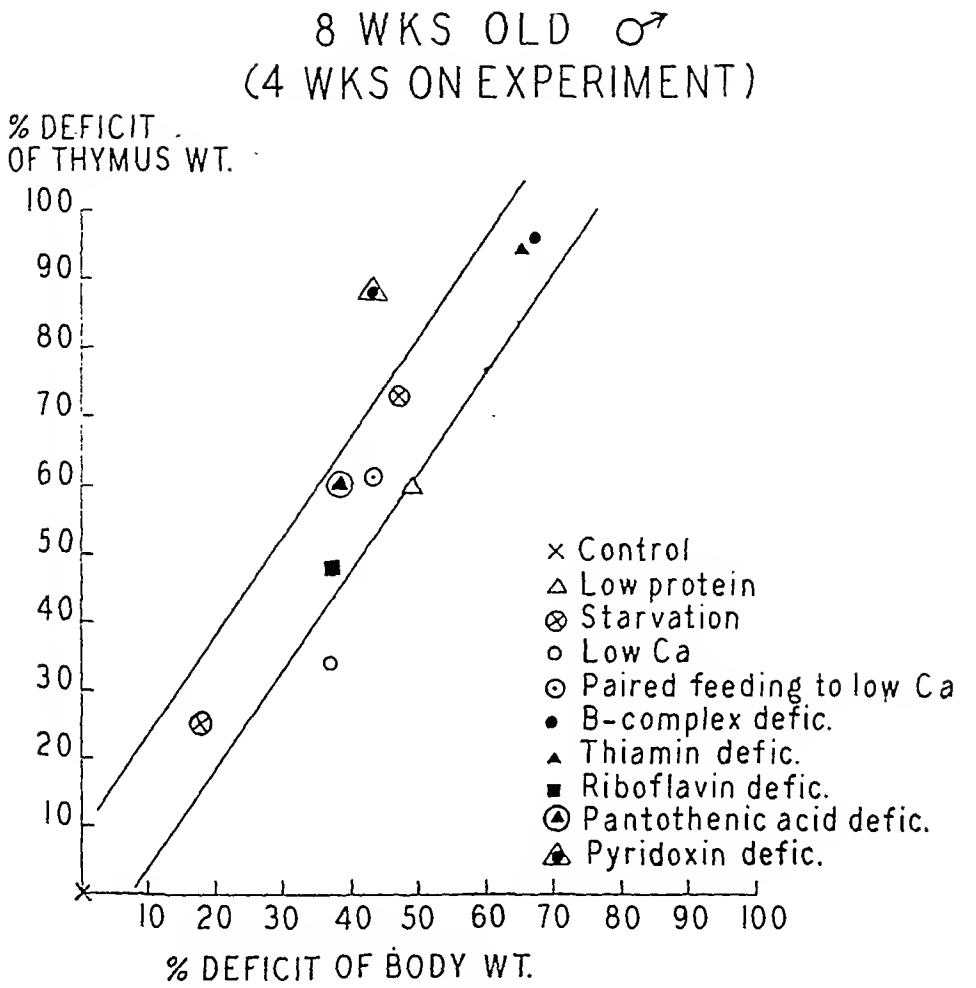


Chart IV.
Relationship between body weight deficit and thymus weight deficit.

growth on a complete ration was considered as an abnormality and thymus weights in such animals were excluded from the use as controls.

The accepted norm of thymus weight was obtained with any of several complete diets, provided that growth was optimal. None of these diets had an apparent effect *per se* on thymus weight.

Chart I presents a log/log plot of thymus weight on body weight of *male* albinos of the Sherman strain from our stock colony as compared with other data from the literature. The band includes the range of our data obtained from 115 animals that grew either optimally or recovered from previous growth disturbance. As reported previously an identical range of thymus weights on body weight was obtained in 56 male rats, castrated or adrenalectomized 3-4 weeks before killing.[6] Jackson and Moment who employed animals growing close to optimally have obtained thymus weights as high as those found in our controls, or even somewhat higher. Fair agreement of all data is evident in the earlier part of the rat's life. The thymus weights from about 100 g of body weight on in Donaldson's (Wistar rat) and Watanabe's data and in our own earlier observations are sig-

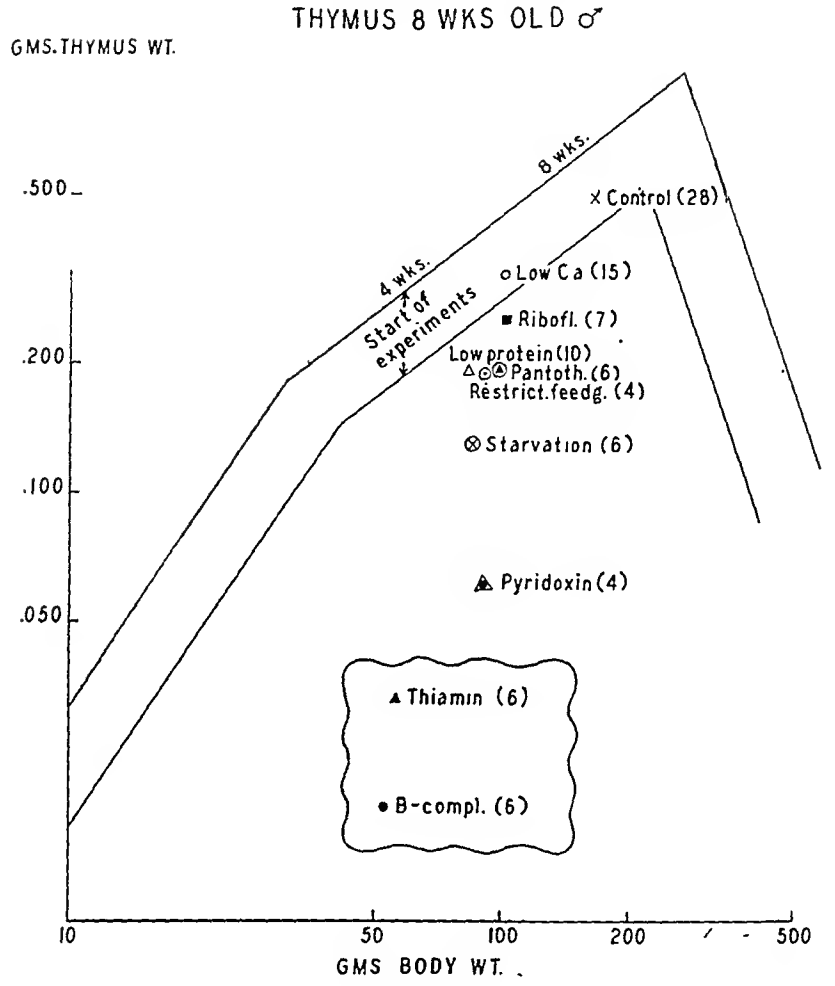


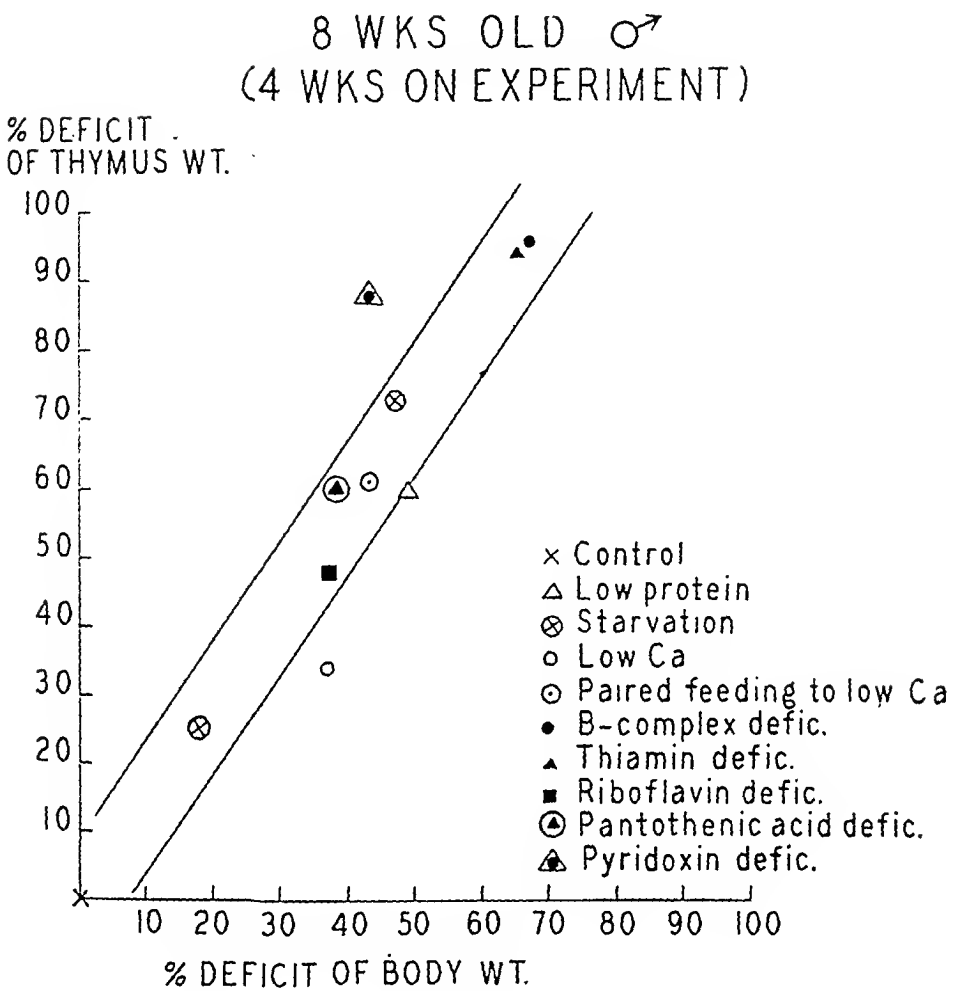
Chart III.

Range of thymus weights obtained from animals growing optimally (Chart I), compared with experimental data.

Recently Zucker and Zucker[7] have shown that in albino rats fed an adequate stock ration, there exists a simple body weight time relation. Their growth formula applied not only to large numbers of rats of their own colony but also to data of others who employed complete diets. This body weight time relationship, obviously cannot be obtained when factors other than dietary interfere with normal growth. Such a factor exists in most rat colonies in form of a large variety of known and unknown diseases.

In the early stages of this investigation a relatively large number of thymus weights was recorded from animals of our stock colony (Sherman strain) kept on a complete ration (Rockland rat diet). Growth records as far as available showed optimal growth only in isolated cases. Later care was taken to improve the living conditions of the animals and to follow their growth regularly. It became evident then that animals showing optimal growth had larger thymus glands than undersized rats. The cause of the growth retardation in the undersized animals was not known but the failure to obtain optimal

[7] Zucker, L., and Zucker, T. F., *J. Gen. Physiol.*, 1942, **25**, 445.



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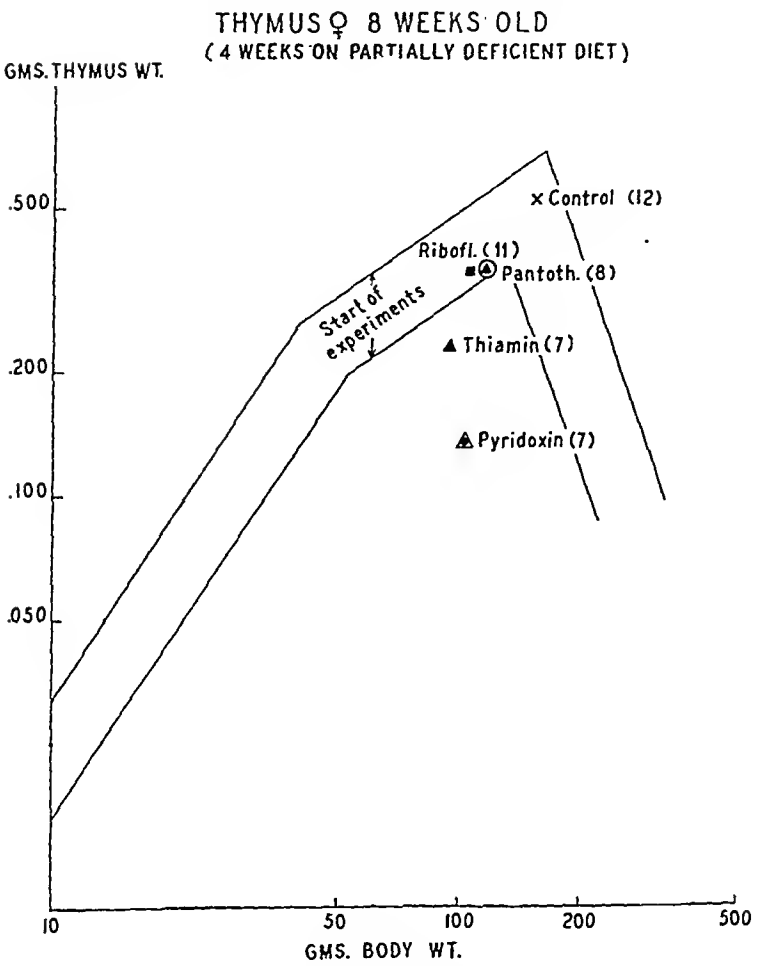


Chart V.

Range of data in Chart II compared with experimental data.[9]

nificantly lower than the accepted norm. In these 3 groups the growth of the animals was below optimal.

Chart II presents a similar plot illustrating thymus growth on body weight in the female albino.

Chart III gives a comparison of experimental data in the 8-week-old male. The band again represents the range of the accepted norm. A number of adverse dietary conditions, mostly produced by the omission of a single substance, from the diet, have been induced for a period of 4 weeks.* It is seen that in most of these conditions the thymus ceased to grow or was slightly atrophic when body growth was retarded. In deficiency of B-complex and in thiamin deficiency growth retardation was marked and was followed in the latter part of the experiment by body weight loss. In these 2 cases, thymic atrophy was extreme. Advanced thymic atrophy without marked effect on body weight was observed only in pyridoxin deficiency. Similar findings in the female rat have been reported previously.[8] No retarda-

* The basic diet was the same as used by Berg, B. N., and Zucker, T. F., Abstracts 107th A. C. S. Meeting, Cleveland, O., April, 1944. The thymi of the animals deficient in single B-factors were kindly given to us by the above authors.

8 Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 151.

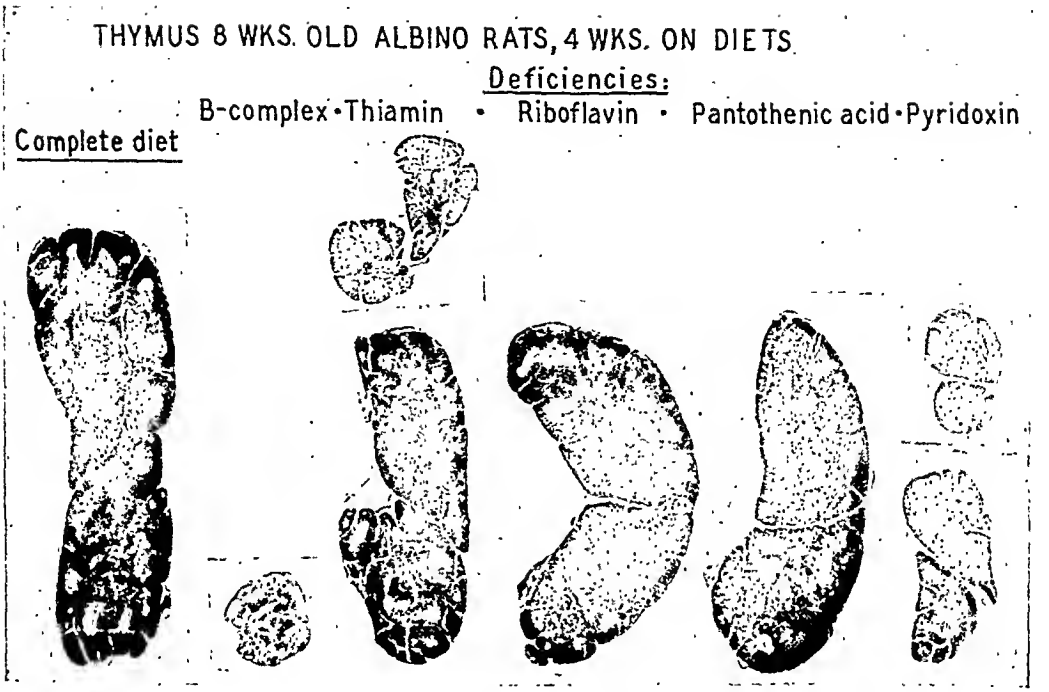


Fig. 1.
The sections of thymus are representative of the glands closest to the average weight in the respective group, except for those in thiamin and pyridoxin deficiency where the smallest and the largest gland in the group have been photographed. (Actual maximal diameter of control gland 10 mm).

tion of thymus growth was evident when body growth retardation and even weight loss was produced by calcium deficiency.

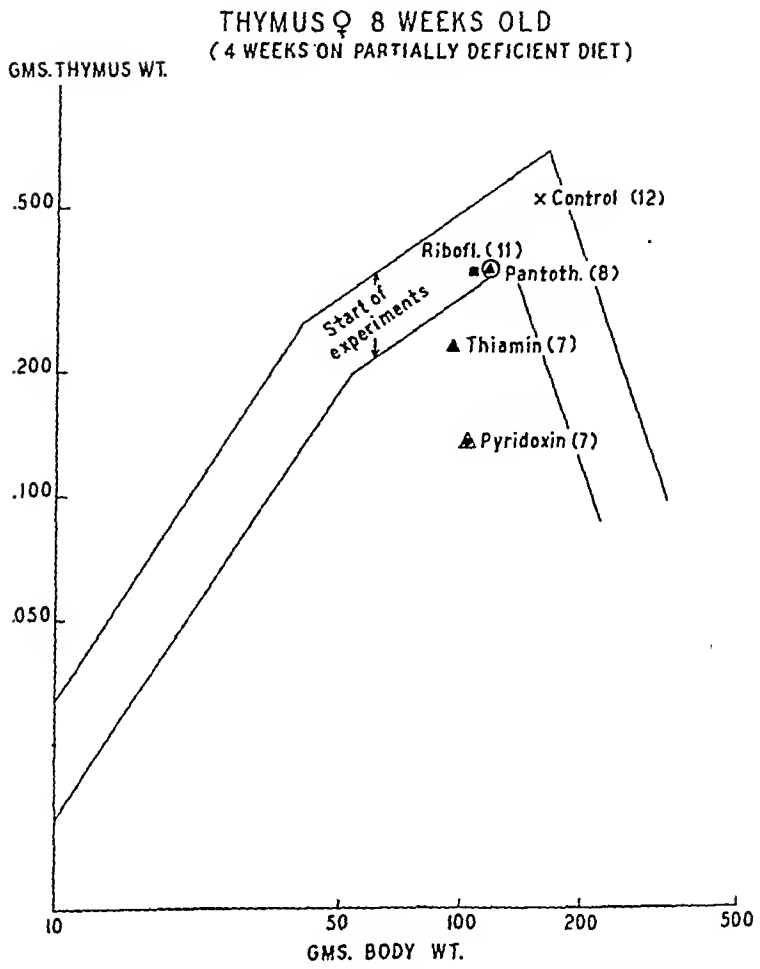
The above data are compared in Chart IV where the thymus weight deficit in percent below that of the controls is plotted against the body weight deficit calculated on the same basis. Roughly most values obtained fit a straight line, the slope of which is about 1.5. Marked deviations from this approximate straight line relationship are evident in 2 cases. In pyridoxin deficiency the thymus weight deficit in relation to body weight deficit was unduly high; in Ca deficiency this ratio was lower than expected. The latter finding is analogous to what is observed on proper comparison, in gonadal and adrenal insufficiency.[6] This occurrence in Ca deficiency is apparently not related to hypocalcemia since parathyroidectomized rats did not show a discrepancy between body weight and thymus weight deficit.[9]

The specific effect of pyridoxine deficiency in producing thymic atrophy is also brought out from the data plotted in Chart V. In this experiment partial deficiencies of single B-factors are compared in female rats. Body growth was almost identical in all groups except that the animals receiving low thiamin lost weight towards the end of the experiment. Actual thymic atrophy was found only in the animals partially deficient in pyridoxin.

In Fig. 1 the histology of glands, of female rats representative of their group, is compared in animals deficient in B-complex, and deficient in single B-factors. It is seen that the marked effect of pyridoxin deficiency on the thymus is also obvious from the morphology. Lymph nodes in these animals were likewise greatly depleted of lymphocytes.

Fig. 2 shows a microphotograph of an atrophic thymus gland representative of those of 10 mice fed a diet deficient in pyridoxin and injected with .2 mg Desoxypyridoxin (kindly supplied by Merck & Co.) 2 times

[9] Unpublished observations.



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nificantly lower than the accepted norm. In these 3 groups the growth of the animals was below optimal.

Chart II presents a similar plot illustrating thymus growth on body weight in the female albino.

Chart III gives a comparison of experimental data in the 8-week-old male. The band again represents the range of the accepted norm. A number of adverse dietary conditions, mostly produced by the omission of a single substance, from the diet, have been induced for a period of 4 weeks.* It is seen that in most of these conditions the thymus ceased to grow or was slightly atrophic when body growth was retarded. In deficiency of B-complex and in thiamin deficiency growth retardation was marked and was followed in the latter part of the experiment by body weight loss. In these 2 cases, thymic atrophy was extreme. Advanced thymic atrophy without marked effect on body weight was observed only in pyridoxin deficiency. Similar findings in the female rat have been reported previously.[8] No retarda-

* The basic diet was the same as used by Berg, B. N., and Zucker, T. F., Abstracts 107th A. C. S. Meeting, Cleveland, O., April, 1944. The thymi of the animals deficient in single B-factors were kindly given to us by the above authors.

[8] Stoerk, H. C., and Zucker, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 151.

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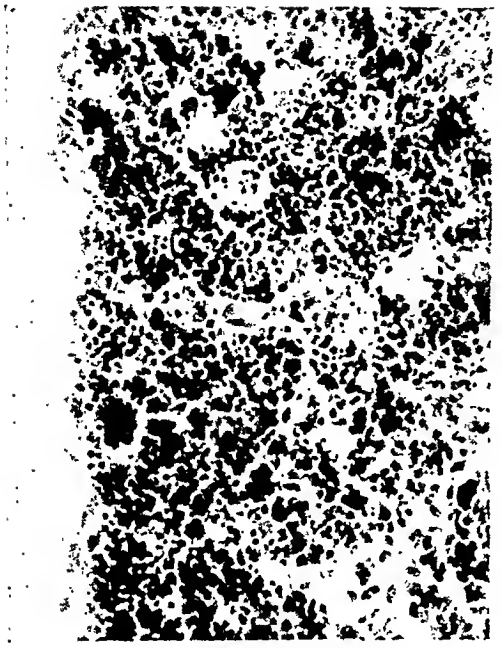

Fig. 2.
Thymus of mouse injected with desoxypyridoxin. Note pycnosis and fragmentation of lymphocytes. The cortical tissue was markedly reduced in amount.

daily over 3 days. The thymi of the injected animals weighed about 50% less than those of non-injected controls. Further studies are necessary to ascertain that no toxic effects are responsible for this change.

The apparently specific effect of even relatively mild pyridoxin deficiency in producing lymphoid atrophy suggests the possibility that pyridoxin may be essential for the maintenance of lymphocytes. This possibility has become of increased interest with the recent finding that a desoxypyridoxin is a powerful inhibitor of B-6.[10] This preparation may therefore perhaps be helpful in the treatment of tumors of the lymphoid tissue. The role recently attributed to lymphocytes in relation to antibodies, and the finding that pyridoxin deficiency suppresses circulating antibodies[11] suggests the possibility that desoxypyridoxin may be used in order to interfere with undesired antibody formation. Investigations in these directions are being carried out.

Summary. In male albino rats (8 weeks of age) exposed to a number of adverse dietary conditions, an approximately linear relationship was found between the amount of body weight deficit and the thymus weight deficit. There were 2 exceptions from this apparent rule: (1) in pyridoxin deficiency, the amount of thymus weight deficit was much greater than expected from the obtained body weight deficit; (2) in calcium deficiency an opposite effect was observed. Here the thymi of undersized animals were of the same weight as those of younger, normal animals of the same body weight.

10 Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 125.

11 Stoerk, H. C., and Eisen, H. N., PROC. SOC. EXP. BIOL. AND MED., 1946, in press.

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Significance of Antigenic Differences Among Strains of Influenza A Virus in Reinfection of Ferrets.*

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The present paper presents data concerning the significance of the antigenic differences among strains of influenza A virus in the reinfection of ferrets. It has been commonly accepted[1-3] that following an influenza virus infection those animals are, for a time thereafter, "solidly immune" to reinfection with that same strain. However, there are few data showing whether or not during the period of "solid immunity" to one strain, ferrets would fail to show clinical manifestations when reinoculated with strains of virus antigenically related to but different from the strain used for the original infection.

Materials and Methods. Ferrets were inoculated intranasally either with influenza A virus or with control material. A febrile reaction following inoculation was taken as the index of clinical infection. Pools of infected allantoic fluid served as the source of virus and uninfected allantoic fluid was used for the control material. The pools of allantoic fluid were collected before the start of the study and were preserved in the CO_2 ice chest until immediately before use. The ferrets were anesthetized by intraperitoneal injections of nembutal. While anesthetized, the animals were bled from the heart and then were given 1.5 cc of inoculum intranasally. In addition to the bleedings made at the time of inoculation, blood was obtained from each animal during the period of convalescence. The virus-neutralizing antibody content of each serum was determined by means of the mouse protection test; the titers are expressed in terms of the initial dilution of serum which protected 50% of the mice from death.[4] All serums showing protection when diluted 1-1024 or more are recorded as having an antibody titer of 2^{10}.

Five groups of 3 ferrets each were used. Three groups (9 animals) were infected with the CC[5] strain of influenza A virus; the 6 remaining animals which were to serve as controls, were inoculated with uninfected allantoic fluid. Six weeks later all of the animals were reinoculated: 3 infected and 3 control animals received the CC strain of virus, 3 infected and 3 controls received the antigenically related WS[1] strain, and 3 infected animals re-

* From the Strain Study Center, Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

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[2] Francis, T., Jr., and Stuart-Harris, C. H., *J. Exp. Med.*, 1938, **68**, 813.

[3] Francis, T., Jr., *J. Exp. Med.*, 1939, **69**, 283.

[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

[5] Magill, T. P., and Sugg, J. Y., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 104.

TABLE I.

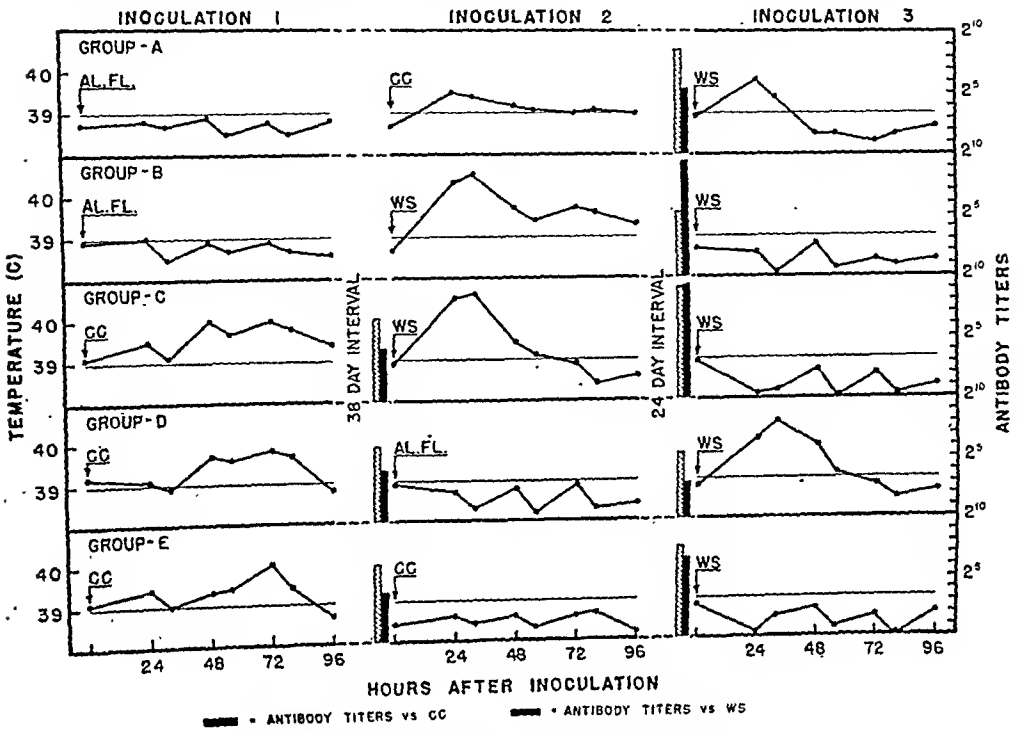
Individual Data, Antibody Titers and Febrile Responses to Reinoculation of Different Strains of Influenza Virus.

		Inoculation 1				Inoculation 2						Inoculation 3					
				Antibody titer 2 wks after inoc.		Antibody titer at time of inoc.				Antibody titer 2 wks after inoc.		Antibody titer at time of inoc.				Antibody titer 2 wks after inoc.	
Group	Ferret No.	Inoculum	Febrile response	CC	WS	CC	WS	Inoculum	Febrile response	CC	WS	CC	WS	Inoculum	Febrile response	CC	WS
A	1	Al. Fl.	—	0	0	0	0	CC	+	x*	x	x	x	x	x	x	x
	2	,,	—	0	0	0	0	,,	+	28.8	24.5	28.8	25.3	WS	+	28.5	27.7
	3	,,	—	0	0	0	0	,,	+	29.5	21.7	28.5	25.3	,,	+	28.5	28.5
B	4	,,	—	0	0	0	0	WS	+	26.5	210	25.5	29.5	,,	—	26.5	29.8
	5	,,	—	0	0	0	0	,,	+	25.5	210	25.5	29.3	,,	—	25.5	29.3
	6	,,	—	0	0	0	0	,,	+	25.5	29.5	24.5	29.5	,,	—	25.5	210
C	7	CC	+	28.5	22.6	26.5	23.3	,,	+	29.5	29.5	28.5	28.3	,,	—	28.5	29.3
	8	,,	+	28.5	24.3	27.2	24.7	,,	+	210	210	29.5	210	,,	—	29.8	210
	9	,,	+	28.3	24.3	26.5	24.5	,,	+	29.5	26.2	29.3	29.3	,,	—	29.5	29.3
D	10	,,	+	28.5	22.8	27.2	25.3	Al. Fl.	—	26.5	24.3	26.5	24.3	,,	+	29.8	210
	11	,,	+	28.8	23.5	26.3	23.3	,,	—	25.5	22.3	25.3	21.5	,,	++	29.5	29.5
	12	,,	+	28.5	23.3	24.5	23.3	,,	—	24.5	22.5	29.7	21.5	,,	+	29.5	210
E	13	,,	+	28.5	24.3	26.5	23.3	CC	—	28.5	27.2	26.7	25.5	,,	—	28.5	29.3
	14	,,	+	28.5	25.3	26.5	24.3	,,	—	28.5	26.5	27.2	25.8	,,	—	28.8	29.3
	15	,,	+	28.5	22.8	26.5	24.5	,,	—	28.5	27.5	28.5	27.8	,,	—	28.5	28.5

* Ferret No. 1 developed an ear infection following inoculation 2 and was discarded.

CHART 1.

Group data. Antibody titers at the time of, and temperature courses following, reinoculation of different strains of influenza virus.



ceived uninfected allantoic fluid. Four weeks after the second inoculation all of the animals were inoculated intranasally with the WS strain.

Results. Temperatures obtained during the 4-day periods following each inoculation, together with antibody titers of the serums obtained at various intervals of time are presented in Chart 1 and in Table I. The results of the neutralization tests with the serums obtained at the time of the first inoculation are omitted because none of those serums possessed demonstrable antibodies against either the CC or the WS strains.

Chart 1 shows graphically, for each of the 5 groups, the average antibody titer at the time of inoculation and the average temperature course following inoculation. Each temperature point and each antibody titer represents the average of data obtained from 3 animals except in the case of Group A, inoculation 3; one of the animals of that group developed an ear infection following inoculation 2 and was discarded.

It is evident (Chart 1) that 4 to 10 weeks following the initial infection ferrets were susceptible to clinical reinfection with influenza virus. The susceptibility to reinfection was influenced by the strain of virus used for reinoculation and by the previous antigenic experience of the animals. Inoculation 2 shows the influence of the strain of virus used for reinoculation upon the febrile responses of ferrets which had had the same previous antigenic experiences. Groups C, D and E all had been initially infected with CC virus. When retested 6 weeks later, the Group C animals, which were inoculated with the antigenically related but different WS strain, gave a pronounced febrile response. On the other hand, the Group E animals, which were retested with CC, showed no more evidence of clinical infection than did the Group D animals which received uninfected allantoic

other, although antigenically related strains.

The results indicate that for a constant amount of virus there was a critical level of specific antibodies which separated a state of relative resistance from a state of relative susceptibility. However, the height of titer at the time of inoculation was not always an index of the resistance of the animal to clinical infection. When ferrets which had approximately the same titers of specific antibodies were inoculated with the same amount of the same virus suspension, the animals which had had the more extensive previous experience with influenza virus strains were likewise the more resistant to clinical infection. The more extensive experience apparently endowed those animals with a more active immunological mechanism which enabled them to mobilize more quickly a higher concentration of strain-specific antibodies. In so far as protection against clinical infection is concerned the protective effect of antibodies is not limited to the quantity present at the time of inoculation but is dependent upon the amount made available before the infecting agent has multiplied sufficiently to evoke clinical manifestations.

Summary. Four to 10 weeks following an initial infection with influenza A virus, ferrets were found to be immune to clinical reinfection with that same strain of virus but were susceptible to reinfection with a strain of virus which was antigenically related to but different from the strain used for the original infection.

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Filamentous Forms of Newcastle Virus.

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Relatively few of the animal viruses have been purified and studied under the electron microscope.[1] This preliminary report deals with the causative agent of Newcastle disease of chickens.

Four strains of virus have been studied, 2 isolated in New Jersey, 2 in California. Strain B was isolated from a field case by Dr. F. R. Beaudette and furnished to us in the 1st embryo passage. Strain W was isolated by us from an outbreak of the disease in a flock of White Leghorns at Bound Brook, N. J. Strain Nap is a laboratory strain isolated by Dr. J. R. Beach in California. These 3 strains are identified as Newcastle virus by their behavior in the developing chick embryo, which they kill with specific hemorrhagic lesions of the brain, feather follicles, and entire embryo in 48 to 84 hours,[2] by their ability to agglutinate chicken red blood cells, by the neutralization of these effects by specific antisera against known strains, and by the reproduction of the natural disease in chickens.[3,4] Strain Cg179, also furnished by Dr. Beach, is highly virulent for chickens and is specifically neutralized by antisera in the embryo.

All four of these strains when partially purified from allantoic fluid by 2 cycles of differential ultracentrifugation show filamentous forms which predominate on the screen of the electron microscope. (Fig. 1 and 2); the phagelike structure is not a constant feature of our preparations, but at no time have we failed to demonstrate filamentous forms. That these forms represent active virus is indicated by their presence in concentrates of the allantoic fluid as early as 24 hours after inoculation until 48 to 72 hours when death of the embryo occurs; by their presence at tem-

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[3] Beaudette, F. R., *Cornell Vet.*, 1946, **36**, 105.

[4] Beach, J. R., *J. Am. Vet. Med. Assn.*, 1946, **108**, 372.

fluid. Inoculation 3 shows the influence of the previous antigenic experience upon the responses of ferrets which were inoculated with the same amount of the same virus suspension. Groups A and D, whose previous influenza virus experience consisted of one inoculation with CC (respectively, 4 and 10 weeks previously), had definite febrile responses following inoculation of WS virus. In contrast, the Group E animals whose previous influenza virus experience likewise had been limited to contact with CC but who had received 2 inoculations of that virus (4 and 10 weeks previously) proved to be clinically immune, as did Groups B and C, which had undergone previous infection with WS.

The influence of the strain of virus used for inoculation, and also the influence of the previous antigenic experience were associated with the height of titer of strain-specific antibodies. Comparison of the antibody titers at the time of inoculation with the subsequent temperature courses shows that all groups which gave evidence of clinical infections when retested (Group C, inoculation 2; and Groups A and D, inoculation 3) had lower average titers of antibodies reactive with the strain used for the reinoculation than did any of the groups found to be immune to clinical reinfection (Group E, inoculation 2; and Groups B, C and E, inoculation 3). The lowest titer of the clinically immune groups occurred in Group E, inoculation 3, and was slightly less than 2^7; the highest titer of the clinically susceptible groups occurred in Group A, inoculation 3, and was slightly more than 2^5. Thus, the group data indicate that there was a critical antibody level which separated a state of relative susceptibility from a state of relative resistance, and that the strain-specific titer at the time of inoculation can be taken as an index of that critical antibody level. However, that titer is not necessarily an index of the total amount of antibodies which will be available to combat clinical infection because the speed with which the animal can produce additional antibodies must also be taken into consideration. That the speed of response is influenced by the extent of previous experience of the animal with influenza virus antigens is indicated by the data of the individual animals (Table I).

Table I shows that Ferrets No. 13 and 14 (Group E), at the time of inoculation 3, had WS antibody titers of $2^{5.5}$ and $2^{5.8}$ but showed no signs of infection following inoculation. In contrast, Ferrets No. 2 and 3 (Group A) both had titers of $2^{5.3}$ against WS yet they gave a mild febrile response following inoculation of that strain of virus. The difference between a titer of $2^{5.3}$ on the one hand and of $2^{5.5}$ or $2^{5.8}$ on the other hand probably is insignificant and hardly can explain the difference in the responses of those animals to inoculation of the WS strain of virus. There was, however, a significant difference in the previous influenza virus experience of these ferrets. The more resistant animals (No. 13 and 14) had had the more extensive experience in that they had had 2 inoculations of the CC strain whereas Ferrets No. 2 and 3 had had only one. That the additional experience conditioned the immunological mechanism to respond more quickly is suggested by the fact that 2 weeks subsequent to inoculation Ferrets No. 13 and 14 had significantly higher WS antibody titers than did Ferrets No. 2 and 3. A sufficiently quick antibody response obviously would have enabled the animals to render the virus ineffective before it had multiplied enough to produce symptoms of clinical infection, irrespective of the antibody titer at the time of inoculation.

Discussion. The present investigation dealt with the resistance of ferrets to clinical reinfection with antigenically related but different strains of influenza virus. The main point was that 4 to 10 weeks following an initial infection the animals, in all cases, were immune to reinfection with the same strain, but were susceptible to reinfection with a strain of virus which was antigenically related to but different from the strain used for the original infection. It would seem, therefore, that the antigenic differences which are known to exist among the A strains of influenza virus are of sufficient magnitude to be of practical importance in problems dealing with immunity to influenza. For example, an immunization procedure might be adequate to produce a high degree of resistance to infection with some strains of influenza virus but afford little protection against infection with

TABLE I.
Purification of Newcastle Virus by Ultracentrifugation.

	Original material			1st supernatant		
	Titer by			Titer by		
Exp. No.	Embryo inoculation	Chicken r.b.c. agglutination	N mg/cc	Embryo inoculation	Chicken r.b.c. agglutination	N mg/cc
1	$10^{9.5}$	1/4000	.48	$10^{6.0}$	1/20	.47
2	$10^{8.5}$	1/800		$10^{6.7}$	1/10	
3	$10^{8.7}$	1/400		$10^{6.3}$	1/10	
4	$10^{8.6}$	1/1600			Undiluted	
5	$10^{8.3}$	1/3200			1/16	
6	$10^{8.7}$	1/800			Undiluted	

	Final concentrate			
	Titer by			
Exp. No.	Embryo inoculation	Chicken r.b.c. agglutination	N mg/cc	Titer/ g protein
1	$10^{10.3}$	1/4000	.014	$10^{15.4}$
2	$10^{8.7}$	1/400	.007	$10^{14.1}$
3	$10^{9.5}$	1/400	.01	$10^{14.8}$
4	$10^{9.6}$	1/800	.008	$10^{14.4}$
5	$10^{9.0}$	1/3200	.004	$10^{14.7}$
6	$10^{8.6}$	1/800	.0036	$10^{14.4}$
			Average	$10^{14.6}$

Virus was titered by calculating the 50% end point mortality after inoculation of 10-11 day chorioallantoic membranes. Since by Avagadro's law a gram of a substance with a molecular weight of 450 million would contain 1.3×10^{15} particles, and since the average preparation here titered to $10^{14.6}$ or 4×10^{14}, there is a three-fold difference between actual and possible end points. The crudeness of the method of titration does not warrant further calculation.

fectivity is lost.

We have not seen these forms in preparations of allantoic fluid containing the same concentration of virus before purification and resuspension in phosphate buffered saline solution. Whether this is due to a masking of form by the allantoic fluid in which the virus exists or whether it is due to a change of shape on transfer into a new medium is undetermined as yet.

Summary. Studies on purified preparations of Newcastle virus of chickens demonstrate that this virus may be filamentous or stringy in form, sometimes with a large head, approaching the tailed forms of the bacteriophages or bacterial viruses.

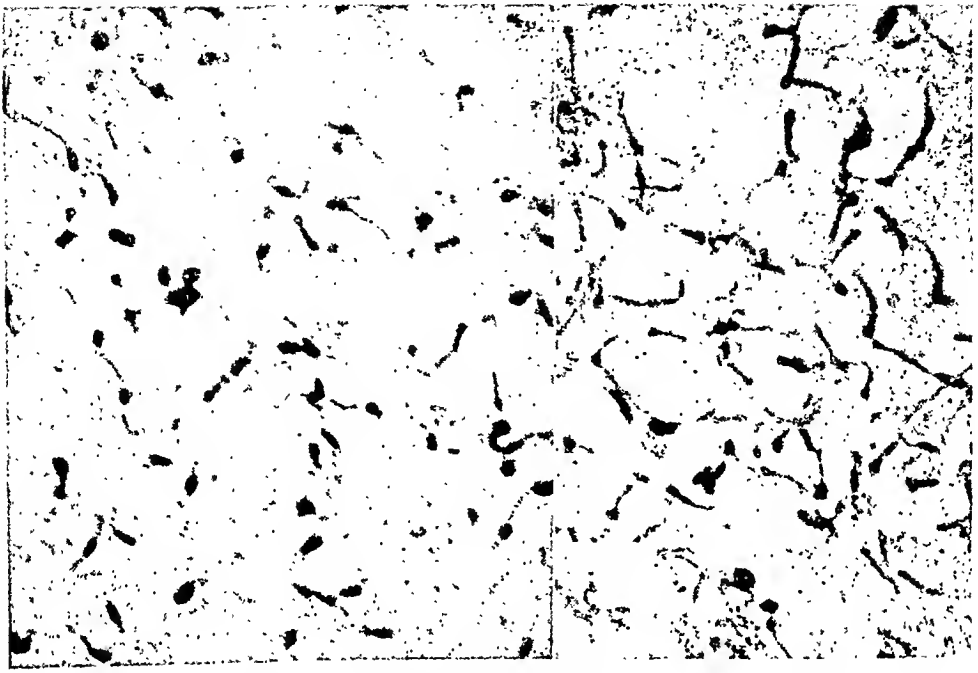

Fig. 1. (Left).
Electron microscope photograph of concentrate of strain B Newcastle virus. 0.01% formaldehyde 2 days. × 17,200.

Fig. 2. (Right).
Concentrate prepared immediately after centrifugation and resuspension in buffered saline. × 17,200.

FIG. 3.
Effect of immune sera. Screen prepared 2 hr after the addition of antisera. Control sera failed to agglutinate virus. × 17,200.

peratures of incubation of the inoculated embryo from 35° to 39°C; by the stability of the virus during centrifugation (see Table I); and by the facts that calculations of size and weight of the virus by electronscope micrography and by light scattering measurements[5] yield a size of about 115 mμ and a molecular weight of 450 million,* which on the basis of demonstrated infectivity for the embryo (Table I) indicates that 10 or less particles are able to infect the embryo; that concentrates of the virus may be specifically precipitated by antisera and electron microscope pictures show the filamentous forms to be clumped by these antisera; and that the sharpness of outline of these forms is gradually lost as the suspension stands and as chick embryo in-

[5] Oster, G., *Science*, 1946, **103**, 306.

* We are indebted to Dr. R. M. Herriott for these determinations.

TABLE I.
Incidence of Deaths in Embryos and Results of Tissue Assays for Virus in Individual Egg Passages.

Passage No.	Route of inoculation	Incidence of deaths in embryos	Tissues assayed	M.I.D.¶ of virus per g tissue
3	C-A*	11/12§		
	Y-S†		Heads	4×10^5
	I-E‡			
			C-A	4×10^5
5	C-A	5/5	Heads	4×10^5
			Viscera	4×10^6
			C-A	4×10^4
	Y-S	4/5	Heads	4×10^6
			Viscera	4×10^7
	I-E	3/4	Brain	4×10^5
6	C-A	5/6	Heads	4×10^6
	Y-S	5/6	Viscera	4×10^7
7	C-A	5/6	Heads	4×10^6
	Y-S	3/4	Viscera	4×10^6
8	C-A	5/6	Heads	4×10^6
	Y-S	6/6	Viscera	4×10^6
10‖	C-A	2/6	Heads	4×10^6
	Y-S	3/6	Viscera	4×10^6
11	C-A	5/6	Heads	4×10^7
12	C-A	11/18	”	4×10^6
13‖	C-A	5/13	”	4×10^5
14	C-A	14/19	”	4×10^5

* Chorioallantoic route.
† Yolk-sac route.
‡ Intra-embryonic route.
§ Denominator = total number of eggs inoculated; numerator, number of deaths in embryos between the 2nd and 5th days.
‖ Inoculated eggs incubated at 37°C, instead of 35°C.
¶ M.I.D. = minimal infectious doses.

During the first 5 passages, groups of eggs were inoculated by one of 3 different routes: (a) chorioallantois according to Burnet,[15] (b) into the yolk sac according to Cox,[16] and (c) intraembryonically according to Elmendorf and Smith.[17] After inoculation, the eggs were incubated at 35°C and were candled daily over a period of 5 days to determine the viability of the embryos. A high per cent became nonviable. A few which became nonviable within 24 hours were discarded. Eggs in which death of the embryo occurred between the 2nd and 5th day were stored in a refrigerator at 4°C. On the 5th day all of the embryos were harvested, including those still viable; the material was pooled and prepared for passage to the next group of eggs. In preparing the material for the next passage, the heads and necks of the individual embryos were pooled and ground in a mortar to a uniform paste. This was emulsified in broth to make a 10% suspension. After centrifugation at 2000 r.p.m. for 10 minutes the supernatant was filtered through a coarse Mandler candle. One-tenth ml of this filtrate served as the inoculum for the next passage.

[15] Burnet, F. M., Medical Research Council, Special Reports Series No. 220, 1936, pp. 1-58.

[16] Cox, H. R., *U. S. Pub. Health Rep.*, 1938, 53, 2241.

[17] Elmendorf, J. E., and Smith, H. H., PROC. SOC. EXP. BIOL. AND MED., 1937, 36, 171.

Cultivation of the Murine SK Strain of Poliomyelitis Virus in Developing Eggs.*

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Dunham and Parker,[1] Gard,[2] and recently Riordan and Sá-Fleitas[3] have reported the cultivation of Theiler's mouse encephalomyelitis virus in developing eggs. Similar attempts to propagate strains of human poliomyelitis virus in eggs have failed;[4-7] a doubtful result, in which one monkey inoculated with egg passage material developed paralysis long after the usual incubation period, has been reported by Gard.[8]

The present report deals with the propagation in fertile eggs of the murine SK strain of poliomyelitis virus described by Jungeblut and Sanders[9] as a strain they had adapted to mice from a monkey passage virus, originally isolated by Trask, Vignec, and Paul[10] from the stools of a child with nonparalytic poliomyelitis. Unlike the mouse-adapted Lansing strain,[11] this strain no longer readily induces typical poliomyelitis in monkeys.[12] It is distinctive also in that it attains high concentrations in mice and is infectious for them by a variety of routes.

Sanders and Jungeblut[13] in 1942 reported the cultivation of this strain in serum ultrafiltrate containing embryonic mouse tissue and made observations on the conditions which favor its growth. The amount of growth was influenced by the relative amount of nervous tissue represented in the cultures and was greatest in the presence of brain tissue. No growth was obtained when embryonic chick tissue was used in place of mouse tissue, and none was obtained in embryonated eggs. Schultz and Irwin[14] were able to confirm the multiplication of this strain in the presence of minced embryo mouse brain tissue and serum ultrafiltrate. The virus was carried through 14 culture passages. It was also carried through a similar number of passages in which minced mouse embryo intestines was used in place of brain tissue. The mouse-adapted Lansing strain of poliomyelitis virus failed to show growth under similar conditions. Chick embryo tissue was not tried.

The present observations were made on fertile eggs incubated for 9 days at 100°F in an egg incubator. The initial inoculum consisted of 0.1 ml of a 10^{-5} dilution of filtered mouse brain suspension, prepared as follows: A 10% mouse brain suspension in broth was filtered through a coarse Mandler candle and diluted serially, in 10-fold dilutions with broth, through to a final dilution of 10^{-7}, counting the unfiltered dilution as 10^{-1}. The 10^{-5} dilution induced infection in 3 out of 3 mice inoculated with 0.025 ml of the dilution intracranially; the 10^{-6} dilution induced infection in 2 out of 4 mice, while 10^{-7} dilution failed to induce infection in all of 5 mice so inoculated.

* Supported by the Howard Frost Poliomyelitis Research Fund.

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14 Schultz, E. W., and Irwin, Elizabeth A., unpublished observations.

TABLE I.
Effect of Biotin and Inositol on Hair Loss (Experiment I).

Lot No.	Ration	Total No. of rats	No. of rats showing hair loss	Avg wt in g after 6 wks (initial wt, 40 g)
1	Soybean oil meal basal	20	13	135
2	S.O.M. basal + 0.1% inositol	32	23	141
3	S.O.M. basal + 0.1% inositol + 4 μg biotin daily (oral)	32	0*	141
4	S.O.M. basal + 0.1% inositol + 4 μg biotin daily (inj)	24	0*	144
5	S.O.M. basal + 0.3% inositol	6	0	132
6	Raw soybean oil meal basal	22	15	94
7	Raw S.O.M. basal + 1 μg biotin daily (oral)	14	2	104
8	Raw S.O.M. basal + 0.3% inositol	8	0	91

* In each of these groups there was one rat which showed early signs of hair loss but spontaneous cure followed.

manner bring out a need for one or both of these vitamins. It is well known that the inclusion of a sulfonamide drug in a purified ration may give rise to biotin and inositol deficiencies.[1,2] The drug probably acts by increasing the demands of the organism beyond its synthetic capacities for the factors or by inhibiting intestinal organisms which ordinarily act as a source of these factors.[3,4] An inositol deficiency has also been produced by feeding a ration composed largely of corn, soybean oil meal and alfalfa.[5] It was suggested that the need for inositol when this natural ration was fed was the result of a vitamin imbalance or of some change in the intestinal flora. A biotin deficiency will also result if a ration containing raw egg white is fed.[6,7] The avidin of the egg white combines with the biotin in the intestinal tract thus forming a complex which is unavailable to the rat.[8]

We have observed an alopecia in rats fed certain soybean oil meal rations which was prevented by supplements of biotin and/or inositol. The condition did not develop when the rations were supplemented with cystine or methionine.

Experimental. Weanling male and female rats of Sprague-Dawley breeding were used in these studies. The soybean oil meal basal ration was composed of soybean oil meal (expeller processed, commercial) 34.0 g, sucrose 57.5 g, salts IV 4.0 g,[9] corn oil 4.0 g, choline chloride 0.3 g, niacin 2.5 mg, calcium pantothenate 1.0 mg, pyridoxine 0.3 mg, thiamine hydrochloride 0.3 mg, riboflavin 0.3 mg, and 2-methyl-1, 4-naphthoquinone 0.1 mg, per 100 g ration. The raw soybean oil meal basal ration differed from this ration in that unheated solvent extracted flakes replaced the soybean oil meal. Two drops of halibut oil were given weekly. Oral biotin supplements were given by dropper, and injected biotin supplements were injected intradermally in saline solution.

Rations were mixed every 2 weeks and were stored at refrigerator temperatures to avoid possible development of rancidity. Rations and distilled water were given daily to insure *ad libitum* consumption.

Results. Experiment I. Table I summarizes growth data and the incidence of alopecia in rats fed the soybean oil meal rations. Only small differences in growth rate

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[6] Boas, M. A., *Biochem. J.*, 1927, **21**, 712.

[7] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

[8] Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **136**, 801.

[9] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

Titrations were carried out on filtrates from each passage.

In assaying the virus content of the embryos from the 5th passage, eggs were separated on the basis of the routes by which the inoculations were made and different portions of the embryos in each group were titrated separately for virus content. These portions included pools of the chorioallantois, pools of the heads of embryos, and pools of the abdominal viscera (kidneys, liver, spleen, stomach and intestine). In those inoculated by the intraembryonic method, pooled brains only were titrated for virus.

After the 5th passage the intraembryonic method was abandoned, and the passages made by the 2 remaining routes were carried independently of each other. In the passages which were made by the chorioallantoic route, the material for the next inoculation was always obtained from the pooled heads of embryos harvested from the previous passage; in those made by the yolk sac route, the material inoculated was always harvested from the pooled viscera of embryos previously infected by the yolk sac route. The purpose of these 2 separate lines of passage was to determine whether the relative difference in the amount of nervous tissue represented would influence appreciably the amount of virus produced.

It has been stated that a high per cent (about 80%) of the embryos died between the 2nd and 5th days. These deaths occurred from the first passage onward. No distinctive gross or microscopic lesions in either the chorioallantois or the embryos have thus far been identified, but this is under further study. The incidence of deaths in embryos in eggs incubated at 37°C was lower (40%) than that in eggs incubated at 35°C.

Our observations are summarized in Table I. Results on certain of the passages have been omitted to save space. These are in agreement with those given. All of the titrations were carried out by inoculating the individual dilutions of the passage material intracranially into groups of 3 to 5 white mice, each with 0.025 ml of the dilution, and 50% infection in the individual groups was counted as the end point.

Neutralization tests against anti-SK serum, prepared before these studies were initiated, were carried out with virus from the 10th egg passage. The filtrate used in the tests titered 10^{-5}, based on 50% infection. It was employed in a dilution of 10^{-4} and 0.5 ml of this dilution was treated with an equal volume of the individual serum dilutions. Complete neutralization was obtained in serum dilutions up to and including 1:512, the highest serum dilution tested. Theiler's GD-VII strain of mouse encephalomyelitis virus was not neutralized by the serum.

Summary. The murine SK strain of poliomyelitis virus was carried through 14 passages on developing eggs without evident diminution of virus content. Its multiplication was associated with a high incidence of deaths in the embryos. The incidence of deaths was influenced by the temperature at which the eggs were incubated after inoculation.†

† Since this manuscript was submitted, we have made observations which indicate that the Lansing strain also, may be cultivated under these conditions.

15472

Alopecia in Rats Fed Certain Soybean Oil Meal Rations.*

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It is generally believed that the rat does not require dietary sources of inositol and biotin when purified rations are fed. However, certain dietary constituents may in some

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TABLE I.
Effect of Biotin and Inositol on Hair Loss (Experiment I).

Lot No.	Ration	Total No. of rats	No. of rats showing hair loss	Avg wt in g after 6 wks (initial wt, 40 g)
1	Soybean oil meal basal	20	13	135
2	S.O.M. basal + 0.1% inositol	32	23	141
3	S.O.M. basal + 0.1% inositol + 4 μg biotin daily (oral)	32	0*	141
4	S.O.M. basal + 0.1% inositol + 4 μg biotin daily (inj)	24	0*	144
5	S.O.M. basal + 0.3% inositol	6	0	132
6	Raw soybean oil meal basal	22	15	94
7	Raw S.O.M. basal + 1 μg biotin daily (oral)	14	2	104
8	Raw S.O.M. basal + 0.3% inositol	8	0	91

* In each of these groups there was one rat which showed early signs of hair loss but spontaneous cure followed.

manner bring out a need for one or both of these vitamins. It is well known that the inclusion of a sulfonamide drug in a purified ration may give rise to biotin and inositol deficiencies.[1,2] The drug probably acts by increasing the demands of the organism beyond its synthetic capacities for the factors or by inhibiting intestinal organisms which ordinarily act as a source of these factors.[3,4] An inositol deficiency has also been produced by feeding a ration composed largely of corn, soybean oil meal and alfalfa.[5] It was suggested that the need for inositol when this natural ration was fed was the result of a vitamin imbalance or of some change in the intestinal flora. A biotin deficiency will also result if a ration containing raw egg white is fed.[6,7] The avidin of the egg white combines with the biotin in the intestinal tract thus forming a complex which is unavailable to the rat.[8]

We have observed an alopecia in rats fed certain soybean oil meal rations which was prevented by supplements of biotin and/or inositol. The condition did not develop when the rations were supplemented with cystine or methionine.

Experimental. Weanling male and female rats of Sprague-Dawley breeding were used in these studies. The soybean oil meal basal ration was composed of soybean oil meal (expeller processed, commercial) 34.0 g, sucrose 57.5 g, salts IV 4.0 g,[9] corn oil 4.0 g, choline chloride 0.3 g, niacin 2.5 mg, calcium pantothenate 1.0 mg, pyridoxine 0.3 mg, thiamine hydrochloride 0.3 mg, riboflavin 0.3 mg, and 2-methyl-1, 4-naphthoquinone 0.1 mg, per 100 g ration. The raw soybean oil meal basal ration differed from this ration in that unheated solvent extracted flakes replaced the soybean oil meal. Two drops of halibut oil were given weekly. Oral biotin supplements were given by dropper, and injected biotin supplements were injected intradermally in saline solution.

Rations were mixed every 2 weeks and were stored at refrigerator temperatures to avoid possible development of rancidity. Rations and distilled water were given daily to insure *ad libitum* consumption.

Results. Experiment I. Table I summarizes growth data and the incidence of alopecia in rats fed the soybean oil meal rations. Only small differences in growth rate

[1] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

[2] Nielsen, E., and Black, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 14.

[3] Woolley, D. W., *J. Nutrition*, 1944, **28**, 305.

[4] Nielsen, E., and Black, A., *J. Nutrition*, 1944, **28**, 293.

[5] Cunha, T. J., Kirkwood, S., Phillips, P. H., and Bohstedt, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 236.

[6] Boas, M. A., *Biochem. J.*, 1927, **21**, 712.

[7] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

[8] Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **136**, 801.

[9] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

TABLE II.
Effect of Cystine and Methionine on Growth and Hair Loss (Experiment II).

Lot No.	Ration	Total No. of rats	No. of rats showing hair loss	Avg wt in g after 6 wk (initial wt, 40 g)
1	Soybean oil meal basal + 0.1% inositol	8	7	132
2	S.O.M. basal + 0.1% inositol + 0.2% *l*-cystine	8	0*	143
3	Raw soybean oil meal basal	16	10	87
4	Raw S.O.M. basal + 0.4% *l*-cystine	8	0*	126
5	Raw S.O.M. basal + 0.4% *dl*-methionine	8	0*	134

* In each of these groups there was one rat which showed early signs of hair loss but spontaneous cure followed.

were observed in the rats receiving the soybean oil meal basal ration and supplemented rations (lots 1-5)). However, 13 of 20 rats receiving the soybean oil meal basal ration (lot 1) developed a marked alopecia. The condition was first observed during the 4th week of the experiment and in all cases was still evident at the end of the 12-week experimental period. The hair loss started in the dorsal region of the head and proceeded bilaterally along the sides to the tail region. All the hair loss reported herein followed this general pattern and persisted throughout the experimental period unless otherwise indicated. The alopecia was not prevented by supplementation with 0.1% inositol (lot 2). The condition was prevented when the basal ration was supplemented with 0.3% inositol (lot 5) or a combination of 0.1% inositol and a daily biotin supplement of 4μg. Oral administration or intradermal injections of biotin were equally effective (lots 3 and 4).

The growth rate of rats fed the raw soybean oil meal basal ration (lot 6) was less than the growth rate of rats receiving the heated commercial soybean oil meal basal ration (lot 1). A slight stimulatory effect on growth was observed when biotin was added to the raw soybean oil meal basal ration (lots 6 and 7). Hair loss similar to that observed in lots 1 and 2 was also noticed in lot 6 when the raw soybean oil meal basal ration was fed. Fifteen of 22 rats in the later group exhibited the characteristic symptoms. Supplementation of this ration with biotin (1 μg per rat per day, lot 7) reduced the incidence of hair loss. Only 2 rats in a group of 14 showed any signs of alopecia. When the raw soybean oil meal basal ration was supplemented with 0.3% inositol (lot 8) no hair loss occurred.

Experiment II. The effects of adding cystine and methionine to the soybean oil meal rations are summarized in Table II. The characteristic hair loss was again observed when the soybean oil meal basal ration was supplemented with 0.1% inositol (lot 1). It also developed when the raw soybean oil meal ration (lot 3) was fed. However, when these rations were supplemented with cystine or methionine no permanent alopecia was noticed. In each of these supplemented groups (lots 2, 4, 5) there was one rat that showed early signs of hair loss which cleared up spontaneously.

Supplementation of the rations with cystine and methionine also resulted in a marked growth stimulus, especially in the case of the raw soybean oil meal rations.

Discussion. Apparently, soybean oil meal in some manner increases the dietary requirement of biotin and/or inositol. This effect may be due to alteration in the intestinal flora, to some absorption disturbance, to a vitamin imbalance or to the action of antivitamins which may be present in soybean oil meal. It is possible that only one of these vitamins is the limiting factor and that the second factor acts only in the formation of the first. It is also possible that both of these factors function in the formation of a third factor which is really the necessary factor. It is interesting that the condition does not develop when the soybean oil meal rations were supplemented with cystine or methionine. It seems possible that inositol

and/or biotin may be closely related to cystine and methionine in normal hair formation.

Another type of hair loss, referred to as the "spectacle eye" condition, has been associated with a biotin deficiency by Neilsen and Elvehjem[10] and to an inositol deficiency by Pavcek and Baum.[11] It seems possible that these two factors may be closely related.

The commercial soybean oil meal rations supported better growth than did the raw soybean oil meal rations. Cystine and methionine supplements stimulated growth especially when added to the raw soybean oil meal rations. These findings support the observations reported by earlier investigators.[12,13] Biotin supplements had a slight stimulatory effect on growth when added to the raw soybean oil meal basal ration. Cunha[14] reported earlier that biotin supplements stimulated growth in rats fed a diet consisting largely of corn, soybean oil meal and alfalfa.

Further investigations now in progress suggest that the situation may become more complicated under certain conditions. While low levels (1-4 μg) of biotin have been shown to prevent the loss of hair, higher levels (12 μg) of this vitamin may actually accentuate the condition. The hair loss resulting from feeding such a high level of biotin was prevented by supplementation with adequate inositol.

Summary. Rats fed certain soybean oil meal rations developed a characteristic hair loss which was prevented by supplementation of inositol and/or biotin. The condition did not develop if the rations contained added cystine or methionine. Inositol supplementation resulted in no marked changes in rate of growth. However, supplements of cystine and methionine markedly increased the rate of growth when added to the raw soybean oil meal basal ration. Biotin supplements had a slight stimulatory effect on growth when added to the same ration. It is believed that soybean oil meal in some manner increases the dietary requirement for biotin and/or inositol. Possible mechanisms for such action may be an alteration of the intestinal flora, some absorptive disturbance, a vitamin imbalance or the presence of antivitamins in the soybean oil meal.

We are indebted to Merck and Co., Rahway, N.J., for synthetic vitamins; and to Abbott Laboratories, North Chicago, Ill., for halibut liver oil.

10 Nielsen, E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 349.

11 Pavcek, P. L., and Baum, H. M., *Science*, 1941, **93**, 502.

12 Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

13 Hayward, J. W., and Hafner, F. H., *Poultry Sci.*, 1941, **20**, 139.

14 Cunha, T. J., Ph.D. Thesis, University of Wisconsin, 1944.

15473

Effect of Low Protein Diets upon Creatine Excretion of the Rat.

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In a previous study[1] qualitative tests upon the urine of rats after a period on a low protein and low choline intake suggested an increased excretion of creatine by these animals. Roberts and Eckstein[2] have shown that the creatine content of the gastrocnemius muscle of young rats is not lowered when a diet deficient in choline and methionine is fed for 3 weeks. A similar finding in chicks was reported by Almquist and coworkers[3] who found a deficiency in dietary choline did not appreciably diminish the muscle creatine.

1 Tidwell, H. C., and Treadwell, C. R., *J. Biol. Chem.*, 1946, **162**, 155.

2 Roberts, E., and Eckstein, H. C., *J. Biol. Chem.*, 1944, **154**, 377.

3 Almquist, H. J., Kratzer, F. H., and Meechi, E., *J. Biol. Chem.*, 1943, **148**, 17.

TABLE I.

Weight changes and Creatine and Creatinine Exeretion of Rats on 5% (Diet 1) and 25% (Diet 2) Protein Diets.

Diet No.		Body wt in grams and % of change. Control	4th da.	8th da.	12th da.	16th da.	20th da.
1	(g)	236	231	226	224	225	226
	(%)	—0.0	—2.1	—4.2	—5.1	—4.7	—4.2
2	(g)	232	239	245	255	260	266
	(%)	+0.0	+3.0	+5.6	+9.9	+12.1	+14.7
		Creatinine excreted—mg/100 g rat.*					
1		3.69 ±.10	3.82 ±.10	3.50 ±.16	3.53 ±.12	3.71 ±.08	3.73 ±.06
2		3.48 ±.12	3.45 ±.10	3.48 ±.10	3.49 ±.08	3.77 ±.08	3.66 ±.09
		Creatine exereted—mg/100 g rat.*					
1		0.61 ±.15	0.40 ±.05	1.18 ±.18	1.32 ±.48	1.19 ±.32	0.62 ±.09
2		0.56 ±.12	0.50 ±.06	0.51 ±.13	0.71 ±.14	0.59 ±.14	0.57 ±.04

* Including the standard error of the mean calculated as follows:

$$\sqrt{\Sigma d^2 / n - 1} / \sqrt{n}$$

These observations along with those that indicate that the physiologically labile methyl groups are preferentially used for growth rather than lipotropism[4] suggest no sparing of these groups as regards the synthesis of creatine.

This study was designed to obtain more information regarding total creatine formation in these rats as measured by its excretion rather than the creatine content of an isolated tissue. Such information would indicate whether the labile methyl groups are conserved in the body when there is an inadequate supply for lipotropic action, and whether the needs for creatine formation take precedence over the needs for lipotropism.

Methods. After 3 weeks on the stock diet (Rockland rat diet), 20 male white rats were divided into 2 equal groups, each averaging about 235 g. They were maintained for 21 days on the special diets containing 5 or 25% casein, 40% Crisco, 5% salt mixture,[5] 2% Cellu flour, and sufficient starch to complete the diet. In addition each rat received one yeast tablet (400 mg) and 2 drops of cod liver oil daily. This diet has been used repeatedly in this laboratory for the production of fatty livers. It seemed possible that the methyl groups required for the synthesis of creatine and choline might be conserved during the acute need for lipotropic substances. If so, this should be reflected in the creatine and creatinine excretion.

The urine was collected under light mineral oil over 24-hour periods while on the stock diet and every 4th day through the 21-day period on the special diets. Intraperitoneal injections of 5 cc of 0.9% saline were given each rat twice daily on the days the urine was collected so as to obtain more suitable urine volumes. The creatine and the creatinine excreted were determined according to the method of Folin[6] with color intensities measured photoelectrically.

Results. More creatinine was excreted per 100 g body weight on the 4th day by the animals receiving the low protein diet and thereafter the amount excreted remained the same during the test period for the animals

4 a. Treadwell, C. R., Groothuis, M., and Eckstein, H. C., *J. Biol. Chem.*, 1941, **142**, 653; b. Treadwell, C. R., Tidwell, H. C., and Gast, J. H., *J. Biol. Chem.*, 1944, **156**, 237.

5 Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, **37**, 572.

6 Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

on both diets (Table I). A significantly greater* amount of creatine was excreted by the animals on the diets deficient in methionine and choline on the 8th and possibly the 12th days only. This increased excretion of creatinine at the start, and of the creatine later, appeared to be associated with the time when the animals were losing weight. Generally an increased creatine excretion was associated with a weight loss in the individual animals whenever it occurred during the experimental period. The differences in creatine and creatinine excreted by the 2 groups were not significant during the latter part of the period when the animals on the low protein diet maintained their weight or gained slightly.

The excretion of similar or greater amounts of creatine or creatinine by the animals on the diet deficient in methionine and choline for lipotropism, gives no indication of a deficiency of methyl groups for creatine formation. This again suggests that there is a preferential use of labile methyl groups for creatine formation associated with the demand for growth. Apparently there is no conservation of the deficient supply of methyl groups during the time fatty livers are developing in these animals, but actually a wastage due to the increased creatine or creatinine excreted when the fed animals are losing weight. The general constancy of the creatinine excretion is in line with the finding of Borsook and Dubnoff[7] that under physiological conditions the phosphocreatine spontaneously yields 2% of free creatinine and that its excretion does not characterize any active process in the tissues. The increased creatinine excretion during the early part of the test period was probably due to a readjustment of the body fluids to the new dietary conditions. The loss of weight involving muscle tissue would explain the increased creatinuria.

Summary. No diminution in the formation of creatine, as measured by its urinary excretion, was found in animals on a diet deficient in labile methyl groups. The available methionine was preferentially used for growth and creatine formation. The increased excretion of creatine by these animals, apparently associated with weight loss, causes a waste of needed methyl groups instead of their conservation.

* Apparent differences were analyzed for significance by the t method of Fisher, R. A., *Statistical Methods for Research Workers*, 7th edition, Edinburgh, 1938. Only those showing a P value of 0.01 or less were considered significant.

Grateful acknowledgment is made to Dr. C. R. Treadwell for his generous advice.

7 Borsook, H., and Dubnoff, J. W., *Annual Review of Biochemistry*, 1943, **12**, 187.

15474

Urinary Secretion of Acetone Bodies in Diabetic Ketosis.

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It is generally recognized[1] that the urine from a patient with diabetic coma, occasionally, may give a negative test for diacetic acid with ferric chloride, and only a mild reaction for acetone bodies with nitroprusside. The studies of Widmark,[2] and Briggs and Shaffer[3] indicated that acetone passes into the urine and expired air by the simple process of diffusion. If this is true, then the concentrations of this fraction of the acetone bodies in the blood and urine should always

1 Joslin, E. P., Root, H. W., White, Priscilla, and Marble, A., *The Treatment of Diabetes Mellitus*, seventh ed., Lea and Febiger Co., Philadelphia, 1940.

2 Widmark, E. M. P., *Biochem. J.*, 1920, **14**, 364.

3 Briggs, A. P., and Shaffer, P. A., *J. Biol. Chem.*, 1921, **48**, 413.

TABLE I.
Concentrations of Acetone Bodies in Blood and Urine from Patients with Diabetic Ketosis.

	Blood Quantitative mg %			Urine				
				Quantitative mg %		Qualitative		
Case No.	Acetone	Total acetone bodies	Glucose	Acetone	Total acetone bodies	$FeCl_3$	Nitro-prusside	Sugar (Benedict's)
1	23.7	90.6	889	22.2	319.2	+++	++++	++++
2	21.5	94.0	507	27.8	436.5	+++	++++	++++
3	25.6	114.0	680	26.2	63.0	Neg.	++	++++
4	29.4	91.0	478	29.7	86.0	Neg.	++	++++
5	33.2	111.0	641	37.6	741.0	++++	++++	++++
6	5.8	28.1	358	5.1	63.3	+	+++	++++
7	6.5	29.6	484	7.7	72.1	++	+++	++++
8	3.0	9.2	390	2.8	36.4	Neg.	+++	++++
9	1.9	4.9	306	2.0	15.2	Neg.	+	++++
10	1.2	5.2	320	1.2	17.8	Neg.	++	++++
11	1.8	3.9	310	2.2	14.8	Neg.	++	+++
12	1.2	3.0	253	1.4	10.6	Neg.	+	+++

be similar; and this relationship should not be influenced by any disturbance of kidney function nor by decomposition of diacetic acid within the urinary passages. However, it has been reported by Martin and Wick[4] that there is no definite relation between the concentrations of acetone in blood and urine with diabetic acidosis; they were inclined to doubt the accuracy of the methods used in early work on the excretion of acetone.

The recently devised method of Greenberg and Lester[5] is especially adapted to the study of this problem, since acetone is determined directly in the presence of diacetic acid. This method was employed in the present study. The volumes of reagents and samples were increased five-fold so that 10 ml of CCl_4 extract were obtained for reading in the Evelyn instrument. Digestions for total acetone were conducted under a condenser with ground glass connections.

Results are presented in the accompanying table of the work done on the admission specimens from a group of patients with diabetic ketosis; the first 5 cases were patients with diabetic coma.

Inspection reveals that the concentrations of acetone in blood and urine are consistently similar. These results, therefore, tend to support the view that acetone is excreted into the urine by the physical processes of filtration and diffusion. This view is also supported by the study of Lehman,[6] which has just appeared. He found similar concentrations of acetone in blood and urine following the intravenous administration of iso-propyl alcohol. Probably the failure of Martin and Wick to observe this relation was due to the fact that what they studied and reported as "acetone" was the fraction of the total acetone bodies precipitated by boiling with mercuric sulfate; this fraction represents acetone plus diacetic acid. It was pointed out by Widmark that the excretion of these 2 substances follows entirely different laws.

Two instances of diabetic coma with "renal block" are provided by Cases 3 and 4. In each case the total acetone body content of the urine was actually less than that of the blood; in each case the urinary test with ferric chloride was negative and that with nitroprusside only 2+. Since the concentrations of free acetone in blood and urine are similar, it would appear that either diacetic or oxybutyric acid is excessively reabsorbed by the renal tubules; the negative reaction with ferric chloride implicates diacetic acid. (The assumption is made that decomposition of diacetic acid in a slightly acid medium, during the flow through the urinary passages,

[4] Martin, Helen E., and Wick, A. M., *J. Clin. Inv.*, 1943, **22**, 235.

[5] Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, 1944, **154**, 177.

[6] Lehman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 232.

is not an important item).

These observations, therefore, indicate that the nature of the renal block is something of a functional disturbance due to an alteration of the tubule threshold for diacetic acid, as was suggested several years ago by Apple and Cooper.[7]

It is not at all clear why this phenomenon should occur in occasional cases. Often it is associated with oliguria, but oliguria is common in diabetic coma, and usually the concentration of diacetic acid in the urine is high. Dehydration and oliguria were more evident in Cases 1 and 2 than in these cases with negative ferric chloride.

[7] Apple, K. E., and Cooper, D. A., *Am. J. Med. Sc.*, 1927, **173**, 201.

Summary. A study has been made of the acetone bodies in blood and urine from a group of patients with diabetic ketosis, including 2 coma patients with urine giving a negative reaction with ferric chloride. The concentrations of free acetone in blood and urine were found to be similar in all cases; this observation supports the view that acetone is secreted into the urine by the physical processes of filtration and diffusion. The observed results were compatible with the suggestion that the cause of a negative urinary reaction with ferric chloride in certain cases of diabetic coma is due to a functional disturbance of the renal tubules, with elevation of the threshold for diacetic acid.

15475 P

Effect of Sulfathalidine and Sulfamethazine on Gaseous Distention in the Obstructed Small Intestine of Cats.

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It has been shown repeatedly that there are 3 sources of gas in intestinal distention (1) swallowed air, (2) decomposition of food, (3) diffusion, chiefly of nitrogen, from the blood into the intestines. According to Fine and his coworkers,[1] the nitrogen from the blood diffuses into the intestine in significant amounts only if other gases have collected in the gut in substantial volume. It has also been shown[1,2] that in the absence of food, swallowed air accounts for most of the gas present in the small intestine. The amount of swallowed air is variable within wide limits but can be controlled in great measure by withholding food and water and an inlying Levin tube. The fermentation or putrefaction of food is the second major source of gas in the obstructed small intestine. The coli-aerogenes-proteus group and certain clostridia, normally present in the small intestine, play a predominant role in this method of gas production. The inhibition of bacterial activity by antibacterial agents should contribute to a more complete control of meteorism. Hence a reduction in the number and metabolic activity of these groups of organisms might have a therapeutic effect.

Many of the sulfonamides now in use have a definite inhibiting action on enterobacteriaceae and clostridia, though each one to a different degree. Sulfathalidine (pthalylsulfathiazole) and sulfamethazine (dimethylsulfadiazine) clinically and experimentally have been shown to be effective against these

[1] Fine, J., and Levenson, W. S., *Am. J. Surg.*, New Series, 1933, **21**, 184.

[2] McIver, M. A., Benedict, E. B., and Cline, J. W., Jr., *Arch. Surg.*, 1926, **13**, 588.

bacteria.[3-6] The following experiments were performed to observe the effect of these drugs on the accumulation of gases in the obstructed small intestine of the cat.

Methods. Cats which had been deprived of food and water for 24 hours were subjected to laparotomy with the usual sterile precautions. Heavy cotton ligatures were used to completely occlude the oesophagus at the cardio-oesophageal junction to eliminate swallowed air and the ileum just proximal to the ileocaecal junction, thus producing a closed loop. Malted milk, which is a rich source of gas in the intestinal tract, was then injected into the stomach. Controls received 100 cc of malted milk without any drug; test animals received the same amount of malted milk containing sulfathalidine or sulfamethazine (1½ g per kg of body weight). At the end of 24 hours all animals were sacrificed and the amount of gas in the obstructed loop was measured by aspirating the entire contents into a syringe. The amount of gas in the stomach and in the small intestine was measured separately. Two animals were found dead at the end of 24 hours and were discarded. All the other animals were apparently in good condition at the time of execution.

A total of 46 animals were studied (Table I)—16 controls, 20 treated with sulfathalidine, and 10 with sulfamethazine.

Results. Gas production in the small intestine is apparently markedly depressed by the chemotherapeutic agents studied. Thus

TABLE I.
Total Gas in Closed Gastro-Intestinal Loops Containing Malted Milk.
A. Sulfathalidine; B. Sulfamethazine; C. No drug.

	A	B	C
	10	30	200
	0	15	75
	10	12	140
	0	0	110
	50	19	136
	0	0	100
	32	5	105
	16	7	200
	55	20	30
	8	12	55
	2		190
	60		130
	20		160
	0		57
	18		62
	3		39
	38		
	10		
	10		
	10		
Average	17.6	12.0	112.9

in the animals receiving sulfathalidine, the total gas production ranged from 0 to 60 cc, in those receiving sulfamethazine, from 0 to 30 cc, and in the control animals from 30 to 200 cc. Only 6 out of 16 controls showed less than 100 cc. These results seem quite significant, particularly considering individual variations in gas production.

This observation is consistent with that of Sarnoff and Fine[7] who showed a protective effect by sulfasuxidine and sulfathalidine on isolated loops of ileum of dogs in which occlusion of the venous return produced gangrene except when these drugs were present in the injured loop.

Conclusions. Gas formation from malted milk in the occluded gastrointestinal tract is significantly depressed by sulfathalidine and sulfamethazine.

3 Poth, Edgar J., and Ross, Charles A., *Texas Reports on Biol. and Med.*, 1943, **1**, 345.

4 Poth, Edgar J., International Abst. Surg., 1944, **78**, 373.

5 Schweinburg, F. B., and Yetwin, I. J., *New Eng. J. Med.*, 1944, **230**, 510.

6 Schweinburg, F. B., and Yetwin, I. J., *J. Bact.*, 1945, **49**, 193.

7 Sarnoff, Stanley J., and Fine, Jacob, *Annals Surg.*, 1945, **121**, 74.

15476

Reduction of General Activity in Male Albino Rats from Electro-Convulsive Shock.

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The use of electro-convulsive shock for the treatment of functional psychoses and certain other abnormal mental states is now widespread. Although its use appears to be well justified on clinical grounds, there is no wholly satisfactory explanation of the physiological changes through which the benefits are effected.[1] It seems probable, therefore, that many types of animal experiments will be needed to supplement human investigations in defining and, ultimately, explaining both the immediate and distant effects of electro-convulsive shock, at the psychobiological level.

Many investigators have noted a reduction in exploratory activity and rate of locomotion of rats in various learning situations, following the administration of electro-convulsive shock. Some of these reports are still unpublished;[2,3] others are summarized by Stainbrook in his recent review.[4] Consideration of these data led Stone[5] to conduct a small-scale study of the effects of electro-convulsive shock on the daily voluntary activity of 6 female rats, housed in the Slonaker-type of activity drum. He found consistent, temporary reductions of activity after shock. The present report is a continuation of this earlier study. Its main objective is to gather confirmatory data from which to make a more reliable estimate of the concurrent effects of electro-convulsive shock on the voluntary activity of male rats than was possible in the preliminary study on females.

Procedure. The convulsive shock was given by applying a 50 milliamperes, alternating current for 0.2 second through electrodes clipped on the ears of the rat. This consistently resulted in a grand mal seizure which started with a generalized tonic contraction of the entire body and then gradually passed into the clonic phase after about 10 seconds. The total period of the seizure proper was between 20 and 30 seconds. In the main, the typical syndrome and its most common variants were like those described in some detail by Golub and Morgan.[6]

The study began with 15 well-tamed males, approximately 70 days old. No shock was administered during the first 25 days of the study as it was desired that the animals become thoroughly adapted to their drums and the routine of handling before the introduction of shocks. From the first day and thereafter each animal was removed from its drum once daily and manipulated as much as would be necessary later on when shocks were to be administered. While the rat was out of the drum the experimenter replenished the supply of food and water, so that a surplus of these was available at all times. As a regular routine each rat was returned to the floor of the drum, ready for activity, whether or not he had been shocked.

Following the 25-day period, in which each rat tended to establish a characteristic level of daily activity, the first electro-convulsive shock was given. One seizure was induced daily in each rat on 5 consecutive days. This course was followed by a 5-day period in which no shock was given. Then followed (1) 2 additional 5-day periods of shock, alternating with 5-day periods of no shock, (2) one 5-day period of pseudo-shock, in which all manipulations except passage of the current occurred, and (3) a 20-day recovery period without

[1] Kalinowsky, L. B., and Hoch, P. H., *Shock Treatments and Other Somatic Procedures in Psychiatry*, New York, Grune & Stratton, 1946.

[2] Horowitz, M. W., and Stone, C. P., *J. Comp. Psychol.*, 1946, in press.

[3] Stone, C. P., and Poplin, Betty, *J. Comp. Psychol.*, 1946, in press.

[4] Stainbrook, E., *Psychol. Bull.*, 1946, **43**, 21.

[5] Stone, C. P., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 150.

[6] Golub, L. M., and Morgan, C. T., *J. Comp. Psychol.*, 1945, **38**, 239.

bacteria.[3-6] The following experiments were performed to observe the effect of these drugs on the accumulation of gases in the obstructed small intestine of the cat.

Methods. Cats which had been deprived of food and water for 24 hours were subjected to laparotomy with the usual sterile precautions. Heavy cotton ligatures were used to completely occlude the oesophagus at the cardio-oesophageal junction to eliminate swallowed air and the ileum just proximal to the ileocaecal junction, thus producing a closed loop. Malted milk, which is a rich source of gas in the intestinal tract, was then injected into the stomach. Controls received 100 cc of malted milk without any drug; test animals received the same amount of malted milk containing sulfathalidine or sulfamethazine (1½ g per kg of body weight). At the end of 24 hours all animals were sacrificed and the amount of gas in the obstructed loop was measured by aspirating the entire contents into a syringe. The amount of gas in the stomach and in the small intestine was measured separately. Two animals were found dead at the end of 24 hours and were discarded. All the other animals were apparently in good condition at the time of execution.

A total of 46 animals were studied (Table I)—16 controls, 20 treated with sulfathalidine, and 10 with sulfamethazine.

Results. Gas production in the small intestine is apparently markedly depressed by the chemotherapeutic agents studied. Thus in the animals receiving sulfathalidine, the total gas production ranged from 0 to 60 cc, in those receiving sulfamethazine, from 0 to 30 cc, and in the control animals from 30 to 200 cc. Only 6 out of 16 controls showed less than 100 cc. These results seem quite significant, particularly considering individual variations in gas production.

TABLE I.
Total Gas in Closed Gastro-Intestinal Loops Containing Malted Milk.
A. Sulfathalidine; B. Sulfamethazine; C. No drug.

	A	B	C
	10	30	200
	0	15	75
	10	12	140
	0	0	110
	50	19	136
	0	0	100
	32	5	105
	16	7	200
	55	20	30
	8	12	55
	2		190
	60		130
	20		160
	0		57
	18		62
	3		39
	38		
	10		
	10		
	10		
Average	17.6	12.0	112.9

This observation is consistent with that of Sarnoff and Fine[7] who showed a protective effect by sulfasuxidine and sulfathalidine on isolated loops of ileum of dogs in which occlusion of the venous return produced gangrene except when these drugs were present in the injured loop.

Conclusions. Gas formation from malted milk in the occluded gastrointestinal tract is significantly depressed by sulfathalidine and sulfamethazine.

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herein described, and the curtailment of exploration and increase of time scores as reported by others[4] are identical in kind. The literature is replete with examples showing that one and the same end-result in terms of reduction of level of activity may be the consequence of many diverse factors. Through factor analysis, van Steenberg[8] found that only a small fraction of the reliability of drum scores can be accounted for by a factor which is common to these and to other performances in mazes, problem boxes, and other tests. Thus, further analyses are needed before one may safely assume that the partial inactivation of rats in the activity drum is effected by the same factors as those which interfere with the exploratory tendency and the increase in time scores on learning tasks.

Summary. A series of daily electro-convulsive shocks in 13 male rats, for 5-day periods, significantly reduced concurrent activity as compared with (1) preshock activity, (2) 5-day periods of no-shock, interposed between periods of shock, and (3) the post-shock recovery period. There is a cumulative effect of successive periods of shock. The mean level of activity reached during the post-shock period of 30 days was significantly lower than that of the preshock period. Whether this difference was due to natural decline of activity with age or to lingering effects of electro-convulsive shock was not determined.

[8] van Steenberg, N. J. F., *Psychometrika*, 1939, **4**, 179.

15477 P

A Second Motor Nerve System to Frog Skeletal Muscle.

S. W. KUFFLER.* (Introduced by R. W. Gerard.)

From the Department of Physiology, University of Chicago.

The existence in skeletal muscle of special tonus mechanisms—particular nerve or muscle fibres or contractile elements—has often been proposed and as often been discarded. Tasaki and Mizutani[1] recently reported that stimulation of small diameter nerve fibres could initiate slow contractions in frog muscles. It appears that no electrical investigation of this problem has yet been made, nor have the structures giving slow contractions been identified. The present investigation following Tasaki and Mizutani's study clearly demonstrates a separate neuromuscular system which may well be involved in tonus and contracture and which has been missed because of the much greater action of the well-known twitch system. This latter has been eliminated (a) by progressive pressure block or galvanic block applied to the frog's sciatic below the point of stimulation following Leksell[2] (the large fibres failing sooner) and then observing various leg muscles, or (b) by cutting all but the desired fibre[1] in a small nerve (about 10 fibres) to an isolated toe muscle (extensor longus digiti IV., 15 mm long, 40 to 60 fibres) observed under the microscope.

As compared with the usual motor nerve fibres, those here active are of smaller diameter, higher threshold, greater resistance to pressure or polarization block and slower conduction. They are probably not autonomic, since they may be larger than 5 micra. A single impulse in one evokes a barely visible movement of an attached small muscle, repeated impulses at 3 to 4 a second give a small local shortening and at 20 to 50 a strong slow local contraction appears and in some preparations an occasional fully-propagated

* Seymour Coman Fellow. The present investigation was aided by grants from the Seymour Coman Fund and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Tasaki, I., and Mizutani, K., *Jap. J. Med. Sciences*, 1944, **10**, 237.

[2] Leksell, L., *Acta Physiol. Scand.*, 1945, **10**, suppl. 31, 84.

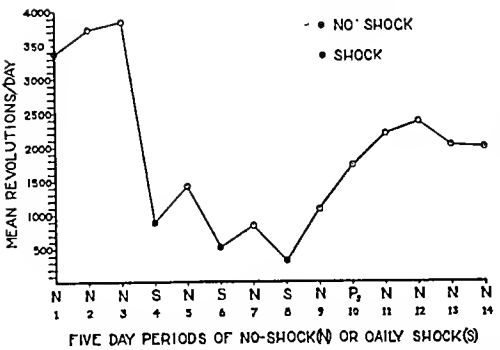

FIG. 1.

Activity of 13 male albino rats in revolving drums, based on the means for 5-day periods. In periods 4, 6, and 8 the rats received a daily electro-convulsive shock.

shock or pseudo-shock. Altogether there were 15 convulsive seizures, spread over a period of 25 days. Two of the rats were eliminated during the experiment. One died, we believe, from a disturbance of the respiratory mechanism. The other became paralyzed in the posterior third of the body, possibly, (but not certainly) from a spinal injury incurred at the time of shock.

Results. The records for the first 10 days of preliminary running were discarded because of the excessive variability of individuals while becoming adapted to the procedures. As shown in Fig. 1, 5-day periods have been numbered serially from 1 to 14. For each period the mean number of drum revolutions per rat per day has been calculated. The significances of differences between group means for the 5-day periods were calculated by use of the conventional small sample technic.

The mean level of activity during the first shock period (number 4) is significantly lower than that of the preshock periods 1, 2 and 3 ($P<0.01$). The same is true of shock periods 6 and 8. The mean of shock period 6 is significantly lower than the mean for no-shock period 5 ($P<0.01$); and the mean of shock period 8 is significantly lower than the means of no-shock periods 5 and 7. Thus, it is clear that the voluntary activity of the group is significantly reduced by 5 daily electro-convulsive shocks whether one considers the level of preshock activity or the levels reached in the intervening no-shock periods.

From period 4 to 8 there is a cumulative downward trend. Its consistency suggests that we have here a real cumulative effect, although the statistical significance of successive differences is less clear-cut than those referred to above (for the differences between periods 4 and 6, $P<0.05$; for periods 6 and 8, $P<0.10$; and for periods 4 and 8, $P<0.02$). In this experiment no rat was consistently hyperactive upon being returned to the drum after being shocked. Indeed, there was much less running on the part of all during the next 6 to 10 hours than had characterized their behavior at comparable times in the preshock period.

The period of pseudo-shock (number 10) is noteworthy because it is attended by a rise rather than a drop in activity. This would seem to indicate that the period was a constituent part of the course of recovery and that the convulsive shock was the essential cause of reduced activity during the periods of shock.

For the last 4 periods of no-shock the mean level of activity is fairly consistent, but lower than that of the preshock periods. Although a few rats were notably hyperactive, more of them were hypoactive, as compared with their preshock records. The net effect is a reduction of the combined mean value for periods 11, 12, 13 and 14 as compared with that of preshock periods 1, 2 and 3 ($P<0.05$). Possibly full recovery of some of the animals had not yet been achieved at the termination of our experiment, 30 days after the last shock. However, we are not warranted in drawing this conclusion without further appraisal of the natural reduction in activity of male rats during the 5th month of age. According to Richter,[7] the mean begins to decline between the 3rd and 4th month. If this should hold for our animals also, it is possible that a part or all of the drop in means for periods 11 to 14, as compared with periods 1 to 3, is due to the natural age decline in drum activity.

Finally, a word of caution should be urged against hasty assumption that specific factors accounting for reduction in drum activity, as

[7] Richter, C. P., *Comp. Psychol. Monog.*, 1922, **1**, pp. 55.

herein described, and the curtailment of exploration and increase of time scores as reported by others[4] are identical in kind. The literature is replete with examples showing that one and the same end-result in terms of reduction of level of activity may be the consequence of many diverse factors. Through factor analysis, van Steenberg[8] found that only a small fraction of the reliability of drum scores can be accounted for by a factor which is common to these and to other performances in mazes, problem boxes, and other tests. Thus, further analyses are needed before one may safely assume that the partial inactivation of rats in the activity drum is effected by the same factors as those which interfere with the exploratory tendency and the increase in time scores on learning tasks.

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[2] Leksell, L., *Acta Physiol. Scand.*, 1945, **10**, suppl. 31, 84.

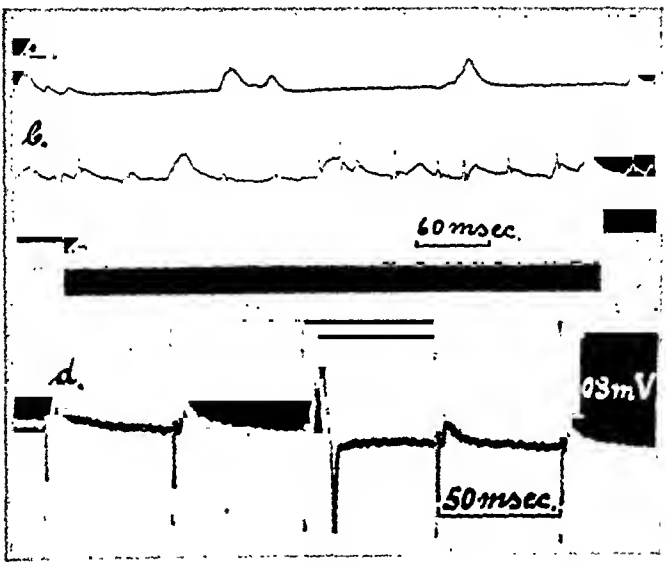

FIG. 1.

a. M. semitendinosus. Spontaneous activity recorded during "rest" in circulated spinal preparation. *b*. Same preparation; activity following on weak reflex excitation (touching of toes). Note increased frequency of local potentials and appearance of some propagated impulses. The latter are accompanied by small twitches. *c*. Activity abolished after cutting nerve supply. *d*. M. extens. long. dig. IV. Potentials set up by stimulation of a single small nerve fiber at 20 per sec. Occasional propagated impulses appeared during this series. Amplification the same in all records.

twitch may also be set up (Fig. 1d). Along with the accompanying potentials the local shortening is limited to the region of the neuromuscular junction.

The local muscle potential resembles the usual endplate potential as obtained after curarization.[3] It appears after a longer interval following stimulation of the nerve, is often slower on rise and fall, and is smaller than the curarized endplate potential. The local potential rises, to a peak of approximately 0.2 mV when excessive short-circuiting is avoided, in 3 to 4 msec. and falls to half in about 13 msec. When recording the activity from the surface of exposed "resting" muscle, local potentials of widely varying time course appear (Fig. 1a). These do not represent the true time course of individual potentials but are a composite effect of multiple scattered units. Repetitive stimulation at 20 to 50 per sec. does not lead to significant local potential facilitation. A similar system of local contractions and potentials has recently been studied in crustacea.[4]

Although the local and propagated muscle responses are so different, the same muscle fibres may be involved in both. The occasional appearance of fully conducted twitch responses with the local potentials on tetanizing of a small nerve fibre has been mentioned (Fig. 1d). Further, dissection of small muscle bundles shows that the muscle fibres are all similar and that the same ones can be excited by "small" and "large" nerve fibres. Both types of nerve endings, therefore, can occur on one muscle fibre. One small nerve fibre can supply multiple groups of endings, each group reaching several muscle fibres in a single area, as revealed by as many as three regions of local potential and shortening.

The local potentials, with or without propagated ones, can easily be obtained from the surface of many leg muscles in the circulated spinal frog. They appear at varying fre-

[3] Eccles, J. C., Katz, B., and Kuffler, S. W., *J. Neurophysiol.*, 1941, 5, 362.

[4] Katz, B., and Kuffler, S. W., *Proc. Roy. Soc.*, 1946, B 133, 374.

quencies (Fig. 1a,b) depending on the number of sensory impulses reaching the cord from the periphery. They can develop tensions of 10 to 15% of a maximal twitch response. Nerve section abolishes them (Fig. 1c). Mammals have not yet been studied.

Summary. A separate small-nerve motor system to frog skeletal muscles is described. It is active reflexly and may be related to muscle tone. It evokes local potentials and local shortening at the region around tbe nerve-muscle junctions, in sharp contrast to the propagated twitches and muscle impulses which can be elicited, by the usual motor nerves in the same muscle fibres. Small fibre activity alone may set up appreciable muscle tension.

I wish to thank Dr. R. W. Gerard for his stimulating interest and help and also Mr. L. Boyarsky for much assistance.

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Benadryl* Fails to Protect Against the Histamine-Provoked Ulcer.†

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It has been shown that synthetic benzhydryl alkamine ethers are capable of preventing fatal asthma induced in guinea pigs by administration of histamine either intravenously or by inhalation of atomized aqueous solution.[1] These drugs also have been demonstrated to be effective in alleviating anaphylactic shock in guinea pigs[2] and dogs.[3] Lowe and his associates also found that the activity of the synthetic benzhydryl alkamine ethers equaled or exceeded that of the 2 Fourneau histamine antagonists, thymoxyethyldiethyl amine (929F) and N-phenyl-N-ethyl-N'-diethylenediamine (1571F).[2] The latter drugs (929F) and (1571F) have been shown to produce no alteration of gastric response to histamine in Heidenhain pouches in dogs.[4,5]

Of the various syntbetic benzhydryl alkamine etbers, β-dimethylaminoethyl benzbydryl ether hydrocbloride (benadryl) was found to be the most potent histamine antagonist in alleviating anaphylactic shock and histamine-induced asthma in guinea pigs.

The effect of benadryl on the action of histamine as judged by blood pressure changes has been studied.[6] It was found that benadryl given intravenously in the amounts of 3 mg per kg body weight abolishes the fall in blood pressure in dogs produced by 0.001 to 0.002 mg per kg body weight of histamine. In that study, the amount of histamine "antagonized" by given doses of benadryl was quantitated and the mechanism of this "antagonism" was stated to be owing to the adsorption of benadryl onto the site of action of histamine, a circumstance which Wells and his associates believes disturbs the histamine equilibrium such that a given amount of his-

* Benadryl was supplied through the courtesy of Parke-Davis Company.

† The researches upon which this presentation is based were supported by the Augustus L. Searle Fund for Experimental Surgical Research, the Citizens' Aid Society, the Robert A. Cooper Fund for Surgical Research, and by grants of the Graduate School of the University of Minnesota.

[1] Loew, E. R., Kaiser, M. E., and Moore, U., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

[2] Loew, E. R., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 235.

[3] Wells, J. A., Morris, H. C., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 104.

[4] Burchell, H. D., and Varco, R. L., *J. Pharm. and Exp. Therap.*, 1942, **75**, 1.

[5] Hallenbeck, C. A., *Am. J. Physiol.*, 1943, **139**, 329.

[6] Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1945, **85**, 119.

tamine has much less opportunity to reach and combine with its site of action.

The effect of benadryl on gastric secretion stimulated by histamine in dogs with Heidenhain pouches has been reported to show a reduction of approximately 40% in gastric secretion in 3 of the 4 dogs studied (8 out of 12 experiments).[7]

It is the purpose of this study to determine whether benadryl is effective in altering the gastric secretory response to histamine, or in protecting against the histamine-provoked ulcer in dogs.

Methods and Experiments. Seventeen dogs were used in 2 series of experiments.

Series I: In the first series 2 Pavlov (innervated) and 2 Heidenhain (denervated) pouch dogs weighing 15 to 30 kg were used. The animals were fasted 18 hours previous to each experiment. Samples of gastric secretion were obtained every 30 minutes for 3 hours after histamine stimulation and studied for volume, free hydrochloric acid, and total acid.

Six sets of observations were made:

(1) Study of the gastric response to 0.5 mg aqueous histamine administered subcutaneously for standardization of the experiments. (12 experiments).

(2) Study of the gastric response to the same dose of histamine subcutaneously, the animals having received benadryl orally in doses of 5 to 10 mg per kg body weight 30 minutes previously. (12 experiments).

(3) Study of the gastric response to the same dose of histamine subcutaneously, the animals having received benadryl subcutaneously in aqueous solution in doses of 10 to 30 mg per kg body weight 20 minutes previously. (24 experiments).

(4) Study of the gastric response to benadryl alone in doses of 5 to 10 mg per kg body weight administered orally and subcutaneously. (8 experiments).

(5) Study of gastric response to histamine-in-beeswax mixture alone (30 mg base) as prepared after the method of Code and Varco[8] administered intramuscularly. (4 experiments). In this way, gastric response over a 24-hour period was studied.

(6) Study of the gastric response to the simultaneous injections of histamine-in-beeswax (30 mg base intramuscularly) and benadryl-in-beeswax mixture (100 mg intramuscularly) prepared in the same manner. (4 experiments).

Series II: Thirteen healthy intact dogs weighing 10 to 25 kg were used.

Seven dogs were given daily intramuscular injections of histamine-in-beeswax (30 mg base) and benadryl-in-beeswax (100 mg) simultaneously several hours after feeding. As controls, 4 dogs were given daily intramuscular injections of histamine-in-beeswax alone (30 mg) and 2 dogs were given daily intramuscular injections of benadryl-in-beeswax alone (100 mg). Animals surviving 40 days were sacrificed at that time; others died or were sacrificed when signs of impending death were present.

Results. Series I: The results of the studies on gastric secretion can be summarized by stating that in 40 experiments benadryl had no demonstrable effect on the secretory response to histamine. There was no evidence of decrease in volume or acidity of the secretion with benadryl premedication. Some of the data indicated that there was a slight increase in quantity and acidity with slight prolongation of the gastric secretion in response to histamine after benadryl administration.

Series II: (Table I). Results of simultaneous daily injections of histamine-in-beeswax and benadryl-in-beeswax show that all (7) dogs developed gastric and/or duodenal ulceration. The 4 dogs receiving histamine-in-beeswax alone developed ulceration. Of the 2 dogs receiving benadryl-in-beeswax alone, one died after 19 daily injections demonstrating early gastric erosions and petechial bleeding points; the other, sacrificed at 40 days, showed no demonstrable pathology.

The 7 animals receiving both drugs were noted to become more debilitated as evidenced by greater weight loss, anorexia and listlessness than those receiving histamine alone. This occurrence also is reflected in

7 Loew, E. R., MacMillan, R., and Kaiser, M. E., *J. Pharm. and Exp. Therap.*, 1946, **86**, 229.

8 Code, C. F., and Varco, R. L., *Am. J. Physiol.*, 1942, **137**, 225.

TABLE I.
Results of Daily Injections of a Mixture of Benadryl-in-beeswax and Histamine-in-beeswax.

Dog No.	Wt in kg	Daily dose of benadryl, mg	Daily dose of histamine base, mg	No. of inj.	Results
1	25	100	30	14	Duodenal erosions
2	20	100	30	5	Three duodenal ulcers, one perforated
3	19	100	30	40	Duodenal ulcer
4	16	100	30	40	" "
5	13	100	30	40	" "
6	10	100	30	29	" " Hemorrhagic gastritis
7	13	100	30	40	Duodenal ulcers
Controls					
8	24	0	30	40	Gastric ulcer Duodenal ulcer
9	23	0	30	40	Gastric ulcer Duodenal ulcer
10	25	0	30	40	" "
11	13	0	30	40	" "
12	20	100	0	19	Gastric erosions Petechial bleeding points
13	15	100	0	40	Negative

the circumstance that only 4 of the 7 dogs survived 40 days, while all 4 receiving only histamine survived 40 days. The 2 receiving benadryl alone survived 19 and 40 days. One dog receiving both drugs died after 5 daily injections of perforated duodenal ulcers with peritonitis. One was sacrificed because of impending death after 14 daily injections of both drugs and showed duodenal erosions, and one sacrificed after 29 daily injections showed duodenal ulcers and hemorrhagic gastritis.

Discussion. Evidence of untoward reactions to benadryl were observed in occasional instances consisting of vomiting when given orally in doses of 10 or more mg per kg of body weight; therefore, we were unable to evaluate the effect of larger doses by this route.

Administration by the subcutaneous route was used to study the effect of larger dosages of benadryl. These dosages were as great and greater than those calculated to be capable of "antagonizing" 0.5 mg of histamine as judged by blood pressure response.

The fact that some of the data (approximately 40%) showed increased volume and acidity where benadryl premedication was given over the response to histamine stimulation alone is not of great significance because the increase was not great. In this respect, benadryl, when given alone, produced neither an increase nor a decrease in the level of gastric secretion in fasting pouch dogs.

The results found in this study, together with the failure of a consistent decrease in gastric secretion as reported by others[7] indicate that benadryl is not a specific antagonist of histamine, but counteracts *some* of the effects of histamine by virtue of its own pharmacological action.

Conclusions. 1. Benadryl fails to alter the gastric secretory response to histamine stimulation in pouch dogs. 2. Benadryl (given in 100 mg doses in beeswax mixture intramuscularly) fails to protect against the histamine-provoked ulcer in dogs.

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Cultivation of *Bacterium tularense* in Peptone Media.*

T. L. Snyder, R. A. Penfield, F. B. Engley, Jr., and J. C. Creasy.
(Introduced by K. H. Lewis.)
From Camp Detrick, Md.

Until recently *Bacterium tularense* has resisted attempts at cultivation in any fluid medium or on any but complex solid media[1] and until recently no dilution plate counting method was described for this organism. Since 1942 there have been described several semi-synthetic liquid media,[2,3] and a more easily prepared fluid medium containing heart infusion broth, hemoglobin, glucose and cystine.[4] Larson[5] has further modified this last medium by replacing the hemoglobin with a completely soluble erythrocyte extract, and has quantitatively confirmed earlier suggestions[2,4] that large inocula are required for propagation in liquid media.

Through a process of elimination and substitution, starting from Rhamy's hemoglobin cystine heart agar[6] we found it possible to derive more simple fluid and solid media quite satisfactory for routine use. These consist of 2% Difco Bacto Peptone, 1% sodium chloride and 0.1% glucose, with or without 2% Difco Bacto Agar. The hydrogen-ion concentration is not adjusted before sterilization. The only precaution that need be carefully observed is the use of relatively large inocula.

The 28 strains which were investigated are listed in Table I in order of decreasing virulence. All strains were originally isolated from human sources and presumably were highly virulent at the time of isolation. Most of the strains, together with information concerning their histories, were supplied us by Professor Lee Foshay. The exceptions are Strain Ince (Professor Cora M. Downs) and Strain 38 (National Institute of Health). The donors' designations are used throughout. Virulence titrations were carried out by injecting groups of 6 mice intraperitoneally with appropriate 10-fold serial dilutions of suspensions of equivalent turbidity (T-500 Fullers earth standard, National Institute of Health). The 50% lethal doses were calculated by the method of Reed and Muench[7] from the percentage mortality within 10 to 14 days, and are recorded as dilutions of the standard suspension. We are indebted to Capt. L. L. Coriell for many of these titres, which are taken from his unpublished data.

Peptone broth was tested for its ability to permit the growth of the 28 strains, using 50 or 100 ml of medium in 250 ml Erlenmeyer flasks. The primary cultures were inoculated into this medium sufficiently heavily from stock blood cysteine agar slants to produce a faint turbidity, and the remaining transfers were made with sterile pipettes. All strains were tested for at least 9 consecutive transfers before terminating the experiment. Twenty-one strains could be maintained by the use of 1% inocula (*i.e.*, one ml of 24-hour broth culture as inoculum for 100 ml sterile broth). A few representative strains of this group (Dieck, Ince and Camp) have been carried through more than 20 transfers, and Strain Schu through 98 transfers, without difficulty. The remaining 7 strains required a larger inoculum for serial cultivation, 10% being successful in all cases. Strain 38, representing this group has been maintained through about 20 transfers under these conditions. With one exception (Strain LR),

* Studies conducted at Camp Detrick, Frederick, Md., between February, 1944, and June, 1945.

1 Francis, E., *J. Bact.*, 1942, **43**, 434.

2 Berkman, Sam, *J. Infect. Dis.*, 1942, **71**, 201.

3 Tamura, J. T., and Gibby, I. W., *J. Bact.*, 1943, **45**, 361.

4 Steinhaus, E. A., Parker, R. R., and McKee, M. T., *U. S. Pub. Health Rep.*, 1944, **59**, 78.

5 Larson, C. L., *U. S. Pub. Health Rep.*, 1945, **60**, 863.

6 Rhamy, *Am. J. Clin. Path.*, 1933, **3**, 121 (cited by *Manual of Dehydrated Culture Media and Reagents*, 7th Edition, Difco Laboratories, Inc., Detroit, Mich., 1943).

7 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Characteristics of Strains Investigated.

Strain	Area and year of isolation	50% lethal dose (ml standard suspension)	Growth with 1% transfers
Chr*	Ohio, 1937	<10-10	+
Gib*	Ohio, 1941	<10-10	+
Ri*	Virginia, 1932	<10-10	+
Holt*	Kentucky, 1944	10-10.6	+
Fox	Ohio, 1940	10-9.8	+
Dieck*	U. S. A., 1944	10-9.6	+
Camp*	Kentucky, 1944	10-9.5	+
Carr*	U. S. A., 1944	10-9.5	+
Ince*	Kansas, 1944	10-9.5	+
Schu*	Ohio, 1941	10-9.5	+
Scherm*	U. S. A., 1944	10-9.2	+
Coll*	U. S. A., 1945	10-8.8	+
Chur	Ohio, 1941	10-8.6	+
LR	Arkansas, 1927	10-8.5	—
Hugh	Ohio, 1940	10-6.3	+
Jap	Japan, 1926	10-4.4	—
HD	Austria, 1935	10-4.0	+
Sto*	Ohio, 1937	10-3.0	+
PF*	Ohio, 1936	10-2.5	+
Russ	Russia, 1928	10-2.0	+
De P	Ohio, 1938	>10-2.0	+
Die	California, 1938	>10-2.0	—
Max	Russia, 1928	10-1.7	—
Pi	Ohio, 1938	10-1.0	+
Ohara	Japan, 1931	10-1.0	—
26	Utah, 1921	10-1.0	—
Li*	Canada, 1934	>10-1.0	+
38	Utah, 1920	avirulent	—

* Yield in peptone broth determined by dilution plate count.

strains requiring an inoculum in excess of 1% were found among those of lesser virulence.

All strains appeared to grow quite as luxuriantly in the 1st and 2nd passages in peptone broth as in later ones, indicating that no process of adaptation was required. The yields of 14 strains (indicated in Table I by asterisks) could be determined by means of a dilution plate counting method described elsewhere.[8] All produced one to 5 billion viable organisms per ml in 24 to 48 hours at 37°C in 50 ml cultures. The effect of aeration on yield was demonstrated with Strains Schu, Camp, Dieck and Ince. An increase in the volume of culture from 50 to 100 ml in 250 ml Erlenmeyer flasks decreased the yield to between 200 millions per ml and 800 millions per ml, while active aeration by means of spargers or by constant shaking increased it to between 3 and 10 billions per ml in 24 hours. Incubation at 23 to 25°C decreased the growth rate, but similar or slightly higher final yields were obtained after 48 to 72 hours. The remaining 14 strains produced roughly equivalent turbidity, but the yields could not be determined by the plate-counting method.

The effect of alterations in the composition of the fluid medium was investigated, using Strain Schu. The average yield was halved by decreasing the peptone concentration to 1%, but some growth occurred with as little as 0.25%. Bacto Peptone could be replaced by Proteose Peptone, Proteose Peptone No. 3 or Bacto Tryptose, but not by Neopeptone or Bacto Tryptone. No difference in yield was observed with 10 different lots of Bacto Peptone. Two and 4% corn steep liquor supported somewhat less luxuriant growth than 2% peptone. Sodium chloride was found to be approximately optimum over the range 0.4 to 2.0% in the presence of one and 2% peptone, but 0.2% gave a poor yield. Glucose was included because there is evidence that

[8] Snyder, T. L., Engley, F. B., Jr., Penfield, R. A., and Creasy, J. C., *J. Bact.*, 1946, 52, 241.

TABLE II.
Effect of Inoculum Size and Reducing Agents on Growth of Strain Schu in Peptone Broth.

Reducing agent	Inoculum (No. of cells per 100 ml flask)	No. of flasks inoculated	No. of flasks turbid after incubation (hrs)						
			16	24	40	48	88	96	336
0	380,000,000	2	2	2	2	2	2	2	2
	38,000,000	3	3	3	3	3	3	3	3
	3,800,000	3	0	0	0	0	2	3	3
	380,000	2	0	0	0	0	0	0	0
0.1% cysteine-HCl	38	3	0	0	3	3	3	3	3
	3.8	3	0	0	3	3	3	3	3
	0.38	3	0	0	2	2	2	2	2
	0.038	3	0	0	0	0	0	0	0
0.01% thioglycollate	38	3	0	0	3	3	3	3	3
	3.8	3	0	0	0	3	3	3	3
	0.38	2	0	0	0	0	0	0	0
	0.038	3	0	0	0	0	0	0	0

it is utilized,[1,9] but our results did not indicate any stimulation by either 0.1 or 1.0%. Variation of the hydrogen-ion concentration over the range pH 6.5 to 7.2 appeared to have no effect.

Only Strains 38, Schu and Chur were tested on peptone agar slants. These strains were twice carried through 20 successive passages without difficulty before terminating the experiment. Transfers were usually made at 24-hour intervals by spreading a 2 mm loopful of growth over the surface of the sterile slant. Growth appeared to originate within streaks of the inoculum, thence spreading over the entire surface. The yield appeared equivalent to that on Rhamy's medium,[6] but was somewhat less than that obtained with blood cystine agar.[10]

The effect of continued cultivation on retention of virulence was investigated only with Strains Schu and Chur. Titrations were carried out as described above, but in this case plate counts made on the same suspensions permit us to report the 50% lethal doses in terms of numbers of organisms. Repeated titration of stock cultures of these strains gave endpoints falling within the range 0.2 to 3.0 organisms. No decrease in virulence was detectable with either strain after 34 passages on peptone agar at 37°C, or with Strain Chur after 30 passages in peptone broth at 37°C. Broth cultures of Strain Schu were studied more extensively. Of 5 separate series, each carried through 50 passages at 25°C, none showed any decrease in virulence, whereas of 5 series carried through 50 passages at 37°C, 3 showed no loss and 2 had decreased to such an extent that the 50% lethal doses were 130 thousand and 11 million organisms respectively.

The minimum effective inoculum for consecutive cultivation in peptone broth was determined more precisely in the case of Strains 38 and Schu, which were selected as representatives of the 2 groups previously described. In this study, the incubation periods were held constant at 24 hours and the inocula varied from 0.1 to 10.0%. Strain 38 could be carried only through one or 2 transfers with 5% or smaller inocula, whereas the control series was maintained through 20 passages with 10% inocula before being discarded. Strain Schu could be maintained indefinitely with 1% inocula, but the 0.3% inoculum series failed to become turbid within 24 hours in the 3rd passage, and the 0.1% series in the second. On the basis of plate counts of 24-hour cultures it was possible to estimate that the minimum inoculum for Strain Schu under these conditions was about one to 4 million organisms per ml of fresh medium.

The constancy of the minimum inoculum size as a characteristic of the strain is open to question. Strain 38 was re-examined after

9 Downs, C. M., and Bond, G. C., *J. Bact.*, 1935, 30, 485.

10 Francis, E., *J. Am. Med. Assn.*, 1928, 91, 1155.

2 years cultivation on blood cysteine agar and still required an inoculum in excess of 1%, but one series of Strain Schu has shown a decrease in the minimum inoculum size to less than 30 organisms per ml after 43 or less passages in peptone broth. This cannot be referred to the effect of a different lot of medium since a control series in the same lot demonstrated the usual requirement for a large inoculum.

Three independent lines of investigation appear to relate the dependence of *Bacterium tularense* on large inocula to an inhibitory action by oxygen or by elevated oxidation-reduction potential of the medium. This is indicated by the effect of reducing agents and decreased oxygen tension on the size of inoculum required, and by the form of growth which occurs in agar shake cultures.

Table II shows the effect of reducing agents. Both cysteine and thioglycollate decreased the minimum effective inoculum of Strain Schu from between 4,000 and 40,000 organisms per ml to approximately one organism per flask. These compounds have also been shown to permit the growth of "strict" anaerobes in fluid cultures exposed to air,[11,12] and to decrease the minimum effective inoculum of certain facultative species.[13]

The effect of oxygen tension was investigated by inoculating peptone agar slants with serial 10-fold dilutions of Strains 38 and Schu and incubating 5 days in mixtures of air and nitrogen. Undiluted commercial nitrogen (assumed to contain 0.5% oxygen) reduced the minimum effective inoculum of Strain 38 to approximately 40,000 per slant, as compared with 4,000,000 per slant in air (21% oxygen). Strain Schu was less affected, the minimum inoculum being reduced from 400 per slant to about 4 per slant, with the optimum at an estimated 1.25% oxygen. These oxygen tensions are within the range which permits the growth of several "obligatory" anaerobes on ordinary media.[14]

In peptone agar shake cultures (0.5 to 1.0% agar) growth of *Bacterium tularense* tended to occur in a narrow zone parallel to, but separated from the surface. This was a constant observation with Strain 38, in which case the zone was always at least 10 mm below the surface and increased in depth as the size of the inoculum was decreased. The zone was about one mm thick after 24 to 48 hours, but increased by extension toward the surface and was several mm thick after a week. Strain Schu demonstrated similar growth stratification with dilute inocula (5000 organisms per ml or less), but larger inocula produced zones which approached the surface as the inoculum increased, until 500,000 to 5,000,000 organisms per ml showed only a one mm thick zone contiguous with the surface. The presence of cysteine caused the growth zone to originate nearer the surface with all inocula, and to extend thereto within 72 hours.

An apparently identical growth formation in agar shake cultures has been described for *Brucella abortus*, and has been ascribed to this species' requirement for carbon dioxide.[15] The preceding experiments on the effect of reducing agents and air-nitrogen mixtures on *Bacterium tularense* cannot lead to a similar conclusion, nor were we able to demonstrate any stimulation by the 2 or 3% carbon dioxide atmosphere obtained by burning a candle in a closed jar. Similar stratification of growth has been observed with a few other bacterial species in ordinary media (see Braun[16] and Prévot[17]) and ascribed to oxidation-reduction potential requirements,[18] and can be induced with numerous facultative anaerobes by the

[11] Quastel, J. H., and Stephenson, M., *Biochem. J.*, 1926, **20**, 1125.

[12] Valley, George, *J. Bact.*, 1929, **17**, 12.

[13] Dubos, René, *J. Exp. Med.*, 1929, **49**, 559; 1930, **52**, 331.

[14] McLeod, J. W., *Acta path. microbiol. Scand.*, 1930, **20** (Suppl. III). 255.

[15] Wilson, G. S., *British J. Exp. Path.*, 1931, **12**, 152.

[16] Braun, H., *Schweiz. Z. allgem. Path. Bakt.*, 1938, **1**, 201, 257, 267; 1939, **2**, 309.

[17] Prévot, A. R., *Ann. Sci. Natur.*, Ser. Bot., 1933, **15**, 23.

[18] Prévot, A. R., *C. R. Soc. biol.*, 1938, **127**, 489.

use of heavy-metal precipitants,[16,19,20] high oxygen pressures,[21] or decreased concentration of nutrients.[22]

The preceding observations appear to relegate *Bacterium tularense* to the poorly defined group of "microaerophiles," as was suggested at one time by Foshay.[23] The species is apparently unusually sensitive to mildly oxidizing intensity in the environment. Our data do not indicate whether this is referable directly to molecular oxygen, or indirectly through the reaction of oxygen with constituents of the medium. As in the case of obligatory anaerobes and of other "microaerophiles," inhibition tends to be abolished by reducing agents, decreased oxygen tension or large inocula. On the other hand, the need for oxygen is indicated by the absence of growth below characteristic levels in agar shake cultures, and stimulation of growth by oxygen is shown by the increased yields obtained in aerated broth cultures.

Summary. 1. *Bacterium tularense* grew luxuriantly and retained virulence during consecutive transfers in peptone broth and on peptone agar without blood or blood constituents. 2. Large inocula were required with the strains tested. The minimum effective inoculum was decreased by reducing agents or by a lowered oxygen tension over the culture, but growth was found to be dependent upon oxygen and stimulated by increased aeration. 3. *Bacterium tularense* appears to be an obligatory aerobe sensitive to mildly oxidizing environment.

19 Burnet, F. M., *J. Path. Bact.*, 1927, **30**, 21.

20 King, J. W., and Rettger, L. F., *J. Bact.*, 1942, **44**, 301.

21 Williams, J. W., *Growth*, 1939, **3**, 21.

22 Cahn-Bronner, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 454.

23 Foshay, Lee, *Am. J. Clin. Path.*, 1933, **3**, 379.

15480

Exudative Trypanosome Pleuritis of Mice Infected Experimentally with *Trypanosoma cruzi*.

MARY N. LEWIS. (Introduced by Robert J. Schnitzer.)

From the Chemotherapy Laboratories, Hoffmann-La Roche, Inc., Nutley, N.J.

In the course of the routine transfer of a strain of *Trypanosoma cruzi* from mouse to mouse it was found that some of the animals developed a pleuritis which seemed to be of specific origin. Since we were unable to find a description of this pathological condition in the literature, our observations are presented in this note.

The strain of *T. cruzi** was carried in mice since August 1942 and was regularly transferred by subcutaneous, occasionally by intraabdominal, injection of blood containing the parasites. The virulence of the strain at the time when the occurrence of pleuritis was first noted had been increased by frequent passages through mice of 3 weeks of age (9-13 g). All the mice injected subcutaneously with 0.05 cc blood containing approximately 50 trypanosomes per microscope field (about 300 × lin. magnification) died within 10-17 days. In general, trypanosomes appeared in the blood of infected animals 2-8 days after inoculation, according to the number of organisms present in the inoculum.

The first observation was made in a young mouse (No. 234) which was inoculated subcutaneously the 28th of May, 1945 and died the 16th of June. At the autopsy ca. 0.5 cc of a clear pleural exudate was found containing 20-30 trypanosomes per microscope field; 0.1 cc of this exudate was injected intraperitoneally into another mouse which showed prostration 13 days later. Its blood contained at this period a very large number of trypanosomes. The animal was sacrificed and

* Strain of the *T. cruzi* (Chagas 1909) was received from the American Type Culture Collection.

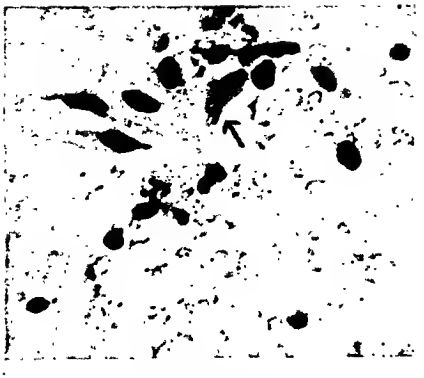

FIG. 1.
Pleural exudate of mouse No. 275, 11 days after subcutaneous infection with *T. cruzi*. Free trypanosome forms. Intracellular leishmania-like bodies in endothelial cells. (←)

the presence of a pleural exudate containing a large number of trypanosomes was noted. No bacteria could be cultured from this exudate on blood agar plates.

A similar observation was made later on in another mouse; No. 247. This animal was infected subcutaneously on the 5th of January, 1946 with 2 drops of blood containing 9 trypanosomes per microscope field. The number of parasites increased progressively in the blood: 6 days after inoculation: 2 trypanosomes per 100 fields; 17 days after inoculation: 4 parasites per one field. The mouse died on the 17th day showing a pleural exudate containing 1-3 trypanosomes per microscope field and a few leishmania-like forms. One drop of the exudate injected subcutaneously to 2 mice lead to a lethal infection. Microscopic sections of the heart and spleen of mouse No. 247 revealed the presence of numerous leishmania-like bodies.

The microphotograph (Fig. 1) of the pleural exudate of a young mouse shows the presence of free trypanosome forms and intracellular leishmania-like bodies. The animal (mouse No. 275; 12 g) died 11 days after subcutaneous infection with an inoculum of 0.05 cc of a suspension containing 50 parasites per field. The number of trypanosomes in the peripheral blood increased from about 14/field on the 3rd day to 160/field shortly before the death. Autopsy revealed the presence of 0.1 cc pleural exudate. Smears of the spleen showed numerous leishmania-like forms. Transfers of the exudate on blood agar did not show growth of bacteria.

In order to obtain information on the incidence of pleuritis in mice infected with *T. cruzi*, 26 young and 9 adult mice were infected subcutaneously. They all showed trypanosomes (2-22 per 100 fields) within 3 days, when the first examination was made. From this group of 35 mice, 25 died 9-17 days after infection, in 14 of them pleural exudate containing a large number of trypanosomes was noted. The volume of the exudate varied between 0.1 and 0.5 cc. Eight of the remaining 10 mice were sacrificed 9-11 days after the infection; in 3 of them pleural exudate with numerous trypanosomes was found. Thus, altogether 51.5% of the 33 mice examined presented pleural exudate containing trypanosomes and leishmania-like forms. In the adult group, 3 out of 9 mice developed pleuritis.

A survey recently conducted by G. Soo-Hoo in this laboratory showed that 18 out of 46 young mice (12-14 g) developed pleuritis (39%) after a heavy infection with a suspension containing approximately 50 parasites per microscope field. Out of another group of 56 mice which died after having received a light infection (5-10 parasites per field) only 2 animals (3.6%) showed exudate in the pleural cavity.

Summary. In the course of the infection of young mice with *T. cruzi* the incidence of pleuritis was observed in 3.6 to 50% of the cases depending on the size of the inoculum. The serous exudate which was sterile if examined with bacteriological methods contained numerous trypanosome forms and intracellular leishmania-like bodies.

use of heavy-metal precipitants,[16,19,20] high oxygen pressures,[21] or decreased concentration of nutrients.[22]

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[19] Burnet, F. M., *J. Path. Bact.*, 1927, **30**, 21.

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[22] Cahn-Bronner, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 454.

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TABLE I.
The Choline Content of Various Materials as Determined by the Reineckate and Neurospora Methods.

Material	Choline content, %	
	By reineckate	By Neurospora*
Purified diet†	<0.03	<0.04
Semipurified diet‡	<0.03	<0.04
Simplified diet§	0.04	>0.08
Peanut meal	0.21	>0.26
Range of 5 practical diets	0.10-0.13	0.11-0.15
Practical diet plus 5% liver meal	0.17	0.26

* The values preceded by the greater-than sign are lower limits of ranges having higher mean values.
† Diet 661 of McGinnis and co-workers.[3]
‡ Diet of Lucas and co-workers.[4]
§ Diet 543 of McGinnis and co-workers.[3]

indicate that, except for those compounds which are determined as choline in the reineckate method, the only compounds of significant effectiveness for Neurospora are mono- and dimethylaminoethanol and monoethylcholine.

On the simplified diet here studied, McGinnis and coworkers[3] observed that choline, methionine, and betaine prevent perosis, but on the purified diet choline only was found to be effective. They concluded that choline *per se* is required for the prevention of perosis. The results of Jukes and coworkers[6,9,10] indicate that mono- and dimethylaminoethanol and mono- and diethylcholine have choline activity for the chick. Mono- and dimethylaminoethanol may function in the chick as choline precursors since these workers found that methionine augmented materially the effects of these compounds.[10]

McGinnis and coworkers[3] have suggested that precursors of choline are present in the simplified diet on which methionine and betaine prevent perosis. Jukes and Oleson[6] have suggested that simple precursors of choline might exist in natural foods. The observations reported in the present paper support these views.

[10] Jukes, T. H., Oleson, J. J., and Dornbush, A. C., *J. Nutrition*, 1945, **30**, 219.

15482

Changes in Alkaline Phosphatase of Kidney Following Renal Damage with Alloxan.

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The alkaline phosphatase which occurs in high concentration in the proximal convoluted tubules of the kidney rapidly disappears during degenerative changes in these structures. The enzyme has been shown to be reduced by nephrotoxic drugs. Alloxan in addition to its injurious action on the islets of Langerhans causes a varying degree of parenchymatous degeneration of the renal tubules and evidence is here reported of an associated depletion of renal alkaline phosphatase in the damaged kidney.

White rats are readily susceptible to alloxan. Bailey, Bailey and Leech[1] produced

[1] Bailey, C. C., Bailey, O. T., and Leech, R. S., *New Eng. J. Med.*, 1944, **230**, 534.

15481 P

Further Evidence for Methylatable Precursors of Choline in Natural Materials.*

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In the course of work on choline metabolism in chicks the choline content of several diets was determined by both a colorimetric method and a microbiological method. The colorimetric method was essentially that of Thornton and Browne[1] using reineckate precipitation. Ethanol was used to extract the choline-bearing substances from the original materials. Hydrolysis was carried out with 3% H_2SO_4 and the precipitation was done after neutralization to pH 6.0 with solid $Ba(OH)_2$ and then to pH 7.5 with 1 N NaOH. The microbiological method was that of Horowitz and Beadle[2] using the Neurospora "cholineless" mutant 34486. The standards as well as unknowns were passed through the adsorption and elution steps. Reproducibility was poor with the Neurospora method on some samples. The results are shown in Table I.

On the purified and semipurified diets the 2 methods were in close agreement. On the practical diet with liver meal and the simplified diet, however, the Neurospora method gave substantially higher values. The simplified diet contained 15% peanut meal.

The value of >0.08% obtained for the simplified diet by the Neurospora method is in essential agreement with that published by McGinnis, Norris and Heuser.[3] The value of <0.04% obtained by the reineckate method is in agreement with a value calculated from the choline contents of the individual ingredients as published by Engel.[5]

The higher value obtained by Neurospora on the practical diet containing liver meal might be expected in view of the results of Jukes and Oleson.[6] These authors found that, if a crude aqueous extract of liver was hydrolized with $Ba(OH)_2$ and then precipitated with reineckate, the resulting filtrate was active for Neurospora.

Jukes and Dornbush[7] observed that dimethylaminoethanol stimulated the mutant Neurospora in a manner similar to choline. Apparently, however, dimethylaminoethanol was not precipitated by reineckate when pure and was only partially precipitated in the presence of choline. These results suggest that those diets on which the higher values were obtained by the Neurospora method in the present study contain dimethylaminoethanol or related compounds. Jukes and Oleson[8] have called attention to the fact that dimethylaminoethanol has been isolated from one member of the Leguminosae.

Numerous compounds were tested with 2 Neurospora "cholineless" mutants by Horowitz, Bonner and Houlahan.[9]. Their data

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† Now with the Institute of Statistics, University of North Carolina, Raleigh, N.C.

1 Thornton, M. H., and Browne, F. K., *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 39.

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Hrs. after injection when rat killed	300 mg alloxan		400 mg alloxan		450 mg alloxan		500 mg alloxan		600 mg alloxan	
	I	II	III	II	III	II	III	II	III	II
6	158	47.5*		24.2	520	23.8	360	22.0	192	16.2
		50.6		24.1		20.8		21.2		16.2
6				22.4		21.7	208	23.8		26.3
				21.7		20.7		25.1		26.9
6				55.2			250	33.0		
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24	174	27.4			500	13.7		12.2	375	12.8
		28.7		23.9		15.0		11.2		13.4
24			280	26.6		20.9			580	27.1
				28.5		26.0				23.9
24			580	28.9	620	29.7				
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26				17.3			305	17.2		
				17.3				18.7		
26			580	19.5			170	16.3		
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28	133	32.6								
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30			215		290	14.2		16.9		
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30				38.0		19.8	224	24.8		
				36.7	580	21.1		25.8		
30			130	21.3			540	16.8		
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48				19.2	230	22.8				
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Normal control	150	40.5		50.0		55.3		52.6	73	44.8
		42.0		52.3						
Normal control				46.6		58.4		62.6	93	53.8
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I Weight of rat in mg.
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a standard in a Fisher electrophotometer. The standard was prepared by adding 15 cc of the 1:3 dilution of the Folin Ciocalteu reagent to 5 cc of a dilute standard phenol solution (containing 0.1 mg of phenol per cc) and diluting to 50 cc. To 4 cc of this mixture was added 1 cc of 20% sodium carbonate and the color allowed to develop for 20 minutes. All tubes were read exactly 20 minutes after the addition of the carbonate. For calculation of the alkaline phosphatase the following formula was used, *viz.*

$\frac{0.04}{RS} \cdot \frac{6}{4} \times 500 \times \frac{RU}{Wt}$ = units of alkaline phosphatase in 1 g of wet kidney; where $\frac{0.04}{RS}$ = number of mg of phenol in 4 cc of standard divided by the reading of the standard on the electrophotometer, (in our experiments RS = 100); $\frac{6}{4}$ = fraction correcting for aliquot used; 500 = dilution of ground kidney; RU = colorimetric reading of unknown kidney material less the colorimetric reading of control; Wt = weight of kidney tissue used.

Results and Discussion. The general response of our animals to alloxan was similar to that reported by other workers. The intensity of the symptoms usually increased

in these animals necrosis of tubular epithelium with a dosage of 200 mg per kg. Dunn and McLetchie,[2] and Goldner and Gomori[3] reported somewhat similar results.

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The technic used for the histochemical preparations was that of the azo dye method,[5] Gomori's method[6] and in many experiments the Kabat and Furth technic.[7] Paraffin sections of kidney tissue fixed in acetone or alcohol were sectioned at 5 μ for routine stains. Sections of both normal and pathologic tissue were affixed to the same slide and subjected to identical procedures in order to obtain comparative values.

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The reduction in renal phosphatase obtained on chemical analysis was paralleled by that found in histochemical preparations. The disappearance of the enzyme following alloxan injection could readily be detected by the naked eye in differences of color intensity occurring in microscopic sections from kidneys of alloxan treated and of normal rats when tissue sections of both affixed to the same slide were subjected to identical technic for the demonstration of alkaline phosphatase. Sections of normal kidney appeared deep gray or black, while those from treated animals were appreciably paler. The loss of enzyme occurred in the luminal border of proximal convoluted tubules and the ascending limb of the loop of Henle, areas in which the phosphatase is localized normally. Frequently in rats receiving 400 mg per kg of alloxan the phosphatase was not only diminished in the luminal border of the tubular cells but was present in large amounts in the mitochondria within the contiguous cells. Hepler, Simonds and Gurley[11] reported a somewhat similar diffusion into these cells following injury by nephrotoxic drugs. This redistribution of the enzyme was not observed with higher alloxan dosage. In a few instances the phosphatase could not be demonstrated histochemically in the proximal convoluted tubules, having been apparently, completely lost from these areas. In preparations of "Zenker" fixed tissue, with a parallel mounting of pathologic and normal sections on the same slide, which were stained with Heidenhain's iron hematoxylin stain, an augmented intensity of black stain occurred in the mitochondria in the kidneys of the alloxan-treated rats as compared to the normal animal. This finding is further evidence of the chemical change produced in the tubular cells by the alloxan. The mitochondria in the pathologic tissue appeared coarser and occasionally in sections which showed marked granular degeneration these structures also had lost their normal parallel alignment and had an irregular distribution.

Three rats which received 400 mg of alloxan per kg were given insulin on the fifth day, with a restoration of the very high blood sugar to a lower level of 200 mg %. The lowering of the hyperglycemia was not, however, accompanied by any apparent improvement in the renal alkaline phosphatase, as chemically and histochemically there was the same low level as in treated rats not receiving insulin therapy. This finding together with the fact that the diabetogenic dosage is lower than that which produces a reduction in the renal phosphatase leads us to believe that 2 different mechanisms are involved in the damage caused by alloxan in pancreas and kidney. The demonstration by Lehman[12] that alloxan in moderate concentrations inhibits the conversion of the Cori ester to the Robison ester and in high concentration sometimes inhibits the formation of both esters may indicate that the alloxan acts *in vivo* by an interference with certain enzymes which differ quantitatively or qualitatively in various tissues.

Summary. The injection of alloxan in dosage of 300 to 600 mg per kg of rat was followed by a definite decrease in the alkaline phosphatase of the kidney. With the 600 mg dosage this enzyme was reduced 50% or more. The decrease in the renal phosphatase obtained by chemical analysis was roughly paralleled by the reduction demonstrated histochemically.

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Renal Alkaline Phosphatase in Rats Dying After Alloxan Injection.

Mg of alloxan injected per kg of rat	Wt in g of rat	No. of hr after injection when rat died	Units of phosphatase (in duplicate) per g of wet kidney
300	165	48	23.9*
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400	82	25	14.7
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400	171	40	13.7
			14.2
450	169	40	14.9
			14.8
450	205	12+	15.1
			15.3
450	139	54	20.4*
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450	131	60	9.6
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450	132	120	19.2*
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* Considerable amount of blood in kidney may be responsible for high values.

with the dosage, although variations occurred in individual rats. Alloxan dosage of 100 mg or less per kg of rat produced practically no symptoms. After a dosage of 200 to 300 mg per kg the animals remained hunched up in their cages for about 24 hours but did not appear ill, and only an occasional rat succumbed to the injection. In 4 to 10 hours after the injection of 400 mg of alloxan per kg the rats appeared listless and ill. After 24 hours they were acutely ill and the excreted urine was tinted pink. Those which survived 30 hours could not stand. Those which received 500 to 600 mg per kg did not live beyond 28 hours, appeared acutely ill and their blood sugars ranged from 200 to 600 mg % at the time of death. The kidneys on excision appeared yellow or a mottled yellow and red. There was a progressive decrease in the survival rate as the dosage increased. Three of the 4 rats receiving 300 mg per kg, 14 of 19 rats of the 400 mg group and only 50% of those which received the 500 or 600 mg dosage survived.

Renal alkaline phosphatase was not decreased when the dosage was 200 mg or less per kg, as shown in 4 groups of rats, each consisting of 4 animals. Rats of each group were given subcutaneously respectively 25, 50, 100 and 200 mg per kg and killed 6, 24, 28 and 48 hours after injection. In all of the treated, as well as the normal control rats, we found the renal alkaline phosphatase to vary between 40 and 51 King and Armstrong units per g of wet kidney. Doses of 300 mg or more per kg caused an appreciable reduction in the phosphatase. The injection of 400 to 600 mg of alloxan per kg was constantly followed by a 50% or greater fall in the enzyme. With high dosage, considerable variation in individual animals in amount of residual enzyme was noted and generally the deleterious effect of the drug was enhanced with prolongation of time after injection up to 21 and 28 hours, when the maximum effect of the drug appeared to be reached. In rats surviving 2 to 3 times this period, little further diminution of phosphatase occurred. The action of the alloxan did not result in the complete removal of the enzyme because phosphatase was present in appreciable amounts in kidneys excised after death from the drug. In 4 rats dying from alloxan the residual enzyme averaged 14.7 units per g of wet kidney, a loss of nearly 70% of the total enzyme as shown in Table II. In 3 of the rats, the values higher than this may have been due to the large amount of blood remaining in these organs after death. In one animal, surviving 60 hours, the phosphatase reached a low level of 9.6 units per g of wet kidney. The water content of these organs was found to be 73 to 77% of their initial wet weight.

The reduction in renal phosphatase obtained on chemical analysis was paralleled by that found in histochemical preparations. The disappearance of the enzyme following alloxan injection could readily be detected by the naked eye in differences of color intensity occurring in microscopic sections from kidneys of alloxan treated and of normal rats when tissue sections of both affixed to the same slide were subjected to identical technic for the demonstration of alkaline phosphatase. Sections of normal kidney appeared deep gray or black, while those from treated animals were appreciably paler. The loss of enzyme occurred in the luminal border of proximal convoluted tubules and the ascending limb of the loop of Henle, areas in which the phosphatase is localized normally. Frequently in rats receiving 400 mg per kg of alloxan the phosphatase was not only diminished in the luminal border of the tubular cells but was present in large amounts in the mitochondria within the contiguous cells. Hepler, Simonds and Gurley[11] reported a somewhat similar diffusion into these cells following injury by nephrotoxic drugs. This redistribution of the enzyme was not observed with higher alloxan dosage. In a few instances the phosphatase could not be demonstrated histochemically in the proximal convoluted tubules, having been apparently, completely lost from these areas. In preparations of "Zenker" fixed tissue, with a parallel mounting of pathologic and normal sections on the same slide, which were stained with Heidenhain's iron hematoxylin stain, an augmented intensity of black stain occurred in the mitochondria in the kidneys of the alloxan-treated rats as compared to the normal animal. This finding is further evidence of the chemical change produced in the tubular cells by the alloxan. The mitochondria in the pathologic tissue appeared coarser and occasionally in sections which showed marked granular degeneration these structures also had lost their normal parallel alignment and had an irregular distribution.

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TABLE I.
Effect of Castration on Toxicity of ANTU in Domestic Norway Rats.

Dose (mg per kg)	No. rats	Age at castration (days)	Age on receiving ANTU (days)	Avg wt	Survival
10	3	C*	132	172	All died
8	2	8	111-128	224	1 survived
8	4	29	117-130	215	2 "
8	5	C*	106-130	191	1 "
6	3	9	127	289	All died
6	3	27	127	262	All "
6	6	C*	119-125	199	5 survived
4	3	8	128	264	2 "
4	5	29	130	194	All "
4	4	C*	127	215	" "
3	2	C*	118	287	" "

*C = Control.

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Table I shows the effect of prepubertal gonadectomy on ANTU susceptibility of rats at ages exceeding 100 days. No difference can be seen between those animals castrated on the 8th and those on the 29th day of age. When the data for the controls are combined an LD_{50} value of 6.7 mg/kg is obtained. It will be noted that deaths occurred among the experimental animals following similar doses. Castration therefore did not produce an increased resistance to ANTU poisoning.

Table II shows the results of the animals injected with either testosterone or estrone for a period of 10 to 19 days before administering ANTU. No significant difference was found between rats receiving the poison on the 20th day after birth and those on the 35th to 40th. All rats (with one exception) of both the experimental and control groups survived on doses of ANTU under 60 mg per kg of body weight. Among the females no difference was found between the experimental rats and their controls, animals of both groups surviving a dose of 50 mg and dying at 70 mg or more. A slight decrease in resistance to ANTU is evident in the male group receiving sex hormone, but they were still very much more resistant than normal mature animals. Among the experimental males 1 out of 4 died upon receiving 50 mg of ANTU per kg and with one exception all 12 died on doses over this. The data for the control males correspond to an LD_{50} of approximately 90 mg/kg.

Discussion. Thiourea and its derivatives presumably cause death in both young and adult rats by increasing the permeability of the lung capillaries, thus causing pulmonary edema and pleural effusion. In young animals either it requires a greater quantity of the drug to change the capillary permeability to the same degree or else the lymphatic mechanism for carrying away the fluid is more efficient in young than older rats. A comparison of the weight of the lungs with that of the body in a series of 100 normal domestic rats from our colony shows no sudden increase in the size of the lungs that might account for the formation of more fluid than the lymphatics are able to cope with. Whether or not the size and number of lymphatic channels increase at the same rate as the lung capillary bed is unknown. This paper shows that changes resulting from the presence of increased quantities of sex hormone in the body during the gradual onset of puberty are not responsible for the change in toxicity. Further investigation is in progress to determine whether other changes at this time controlled, either directly or indirectly, by the pituitary are responsible. These include growth of the animal, involution of the thyroid, and changes in metabolic rate.

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15483

Relation Between Sex Hormones and Changes in Susceptibility of Domestic Norway Rats to Alpha-Naphthyl Thiourea.*

PHILIP V. ROGERS.† (Introduced by Curt P. Richter.)

From the Psychobiological Laboratory, Phipps Psychiatric Clinic, The Johns Hopkins Hospital, Baltimore, Md.

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The experiments referred to above indicate but do not prove any direct connection between sexual maturation and susceptibility to poisoning by thiourea and ANTU. The present study, therefore, was undertaken to discover whether actual pubertal changes are responsible for this great difference in susceptibility or whether the coming into puberty at about the same time is purely coincidental. To do this, gonads were removed from one group of young rats to determine if the resistance to ANTU would remain prepubertal even after they had attained the age and weight of adult animals. A second group received sex hormone (testosterone or estrone according to the sex) in order to develop the secondary sex organs at an earlier age than normal and thus to determine whether the response to ANTU poisoning would be that of a young prepubertal rat or that of an adult.

The first part of this experiment included 40 albino rats. As it has been shown that there is no significant sex difference in resistance to acute ANTU poisoning,[1] male litter mates were used as controls for operated females and vice versa. Twenty animals were gonadectomized, the remaining 20 serving as controls. All operations were performed under ether anesthesia on either the 8th or 29th day of life (3 on the 27th). When both experimental and control animals were over 100 days old they were given intraperitoneal injections of ANTU in olive oil. The doses were adjusted so that each animal received 1/10 cc of oil suspension per 100 g body weight. Autopsies performed on all experimental animals revealed no residual gonadal tissue.

Twenty-nine experimental and 33 litter mate control animals were used in the second part of the study. One-half milligram of testosterone propionate (Perandren) was injected daily into the experimental males and 100 I.U. estrone (Theelin) in oil into the females. Injections were started from the 14th to the 22nd day of age and continued for from 10 to 19 days. At the end of the injection period both experimental and control animals were given ANTU in olive oil in the same manner as the first group. At autopsy the condition of the secondary sex organs was checked in all animals to make certain that the injected sex hormone had been effective. Rats used for these experiments came from a colony originally obtained

* This work was carried on under a contract between the Chemical Warfare Service of the U. S. Army and The Johns Hopkins University.

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4 Drinker, C. K., *Pulmonary Edema and Inflammation*, Cambridge, Harvard University Press, 1945, p. 39.

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8	4	29	117-130	215	2 "
8	5	C*	106-130	191	1 "
6	3	9	127	289	All died
6	3	27	127	262	All "
6	6	C*	119-125	199	5 survived
4	3	8	128	264	2 "
4	5	29	130	194	All "
4	4	C*	127	215	" "
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50	1	♀	21	33		10	Survived
50	2	♀	40	88		19	"
50	2	♂	35	55	19		1 "
50	2	♀	39	75		C*	"
50	3	♂	41	85	C*		"
60	2	♀	37	77		10	Died
64	1	♀	33	55		19	Survived
64	2	♀	35	54		C*	"
64	1	♂	35	58	C*		Died
70	2	♀	37	69		19	"
75	1	♀	24	37		10	"
75	2	♀	40	82		10	"
75	2	♂	24	32	10		"
75	1	♂	37	75	10		"
75	2	♂	35	52	19		"
75	1	♀	40	92		C*	"
75	1	♂	26	38	C*		"
75	5	♂	40	80		C*	Survived
100	2	♂	40	94	10		Died
100	2	♂	21	34	19		"
100	4	♂	38	71	19		2 survived
100	1	♀	38	48		19	Died
100	2	♂	26	36	C*		"
100	4	♂	41	78	C*		3 survived
100	1	♀	40	89		C*	Died
125	1	♂	40	69	10		"
125	1	♀	41	48		C*	"
125	1	♂	41	86	C*		"

* Control.

died from doses in the same range as the controls. A second group of 29 suckling rats received daily injections of testosterone or estrone. Both experimental animals and their controls were then given ANTU when they were 35-40 days old. No significant difference in the susceptibility was found. It is, therefore, concluded that the onset of sexual maturity coincides with the decrease in resistance to alpha-naphthyl thiourea but does not produce it.

15484

IV. Effect of Subtilin on the Course of Experimental Anthrax Infections in Guinea Pigs.*

A. J. Salle and Gregory J. Jann.

From the Department of Bacteriology, University of California, Los Angeles.

In previous communications[1,2] it was reported that subtilin showed an extremely low toxicity index to living embryonic chick heart tissue fragments cultivated *in vitro* and that it exerted a powerful *in vivo* action on the course of experimental pneumococcus Type III infections in mice. Animals treated with subtilin 9 hours after being injected with the organism were quickly cured of the infection. The antibiotic did not exhibit any apparent toxic reaction in the mice.

Subtilin is effective chiefly against Gram-positive bacteria *in vitro*.[3] A few Gram-negative organisms are equally as susceptible. However, the great majority of Gram-negative organisms are not susceptible unless considerably higher concentrations are used. *Bacillus anthracis* is Gram-positive and easily destroyed *in vitro*.

In the present communication, results are given on the effectiveness of subtilin on the course of experimental anthrax infections in guinea pigs.

Experimental. Two guinea pigs weighing approximately 500 g each were injected intraperitoneally with 0.1 cc of a 24-hour nutrient broth culture of *Bacillus anthracis*. One animal was not treated but served as the control; the other was treated immediately with 3 cc of a solution containing 2 mg subtilin per cc (total of 60 units[1]) and at stated intervals thereafter. The subtilin was dissolved in a 5% solution of glucose and all injections were made intraperitoneally. The treatments were discontinued after the 4th day and the animal kept under observation. The schedule of treatments and results are recorded in Table I.

TABLE I.
Treatment of Anthrax in Guinea Pigs.

Date	Time	Control animal	Treated animal
2-14-46	12:30 PM	0.1 cc culture	0.1 cc culture
	" "	—	3 cc subtilin
	3:30 "	—	3 " "
	6:00 "	—	3 " "
	11:00 "	—	3 " "
15	2:00 AM	—	3 " "
	4:30 "	—	3 " "
	6:30 "	—	3 " "
	9:30 "	—	3 " "
	12:30 PM	—	3 " "
	3:30 "	—	3 " "
	9:30 "	—	3 " "
16	1:30 AM	—	3 " "
	4:30 "	—	3 " "
	9:30 "	—	3 " "
	3:00 PM	—	3 " "
	10:00 "	Animal died	3 " "
17	10:30 AM		3 " "
	8:00 PM		3 " "
18	10:30 AM		3 " "
19	3:30 PM		Treatments discontinued
28	12:00 M		Animal died

It may be seen that the control animal (untreated) died in less than 72 hours. The treated animal was given subtilin for 4 days, then observed for an additional 10 days (total 14 days) when death occurred.

Since the culture of *Bacillus anthracis* contained many spores, it is believed that these must germinate into vegetative cells before they can be destroyed by the subtilin. It would appear that some spores did not germinate until after treatment was discontinued.

A second experiment was performed in which 4 guinea pigs were treated instead of only one, and the treatments continued for a longer period of time. The animals were each inoculated with 0.1 cc of a 24-hour nutrient broth culture of the organism, and varying periods of time were allowed to elapse

* This investigation was aided by a grant from Eli Lilly and Company, Indianapolis, Ind. The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, California.

1 Salle, A. J., and Jann, G. J., Proc. Soc. Exp. Biol. and Med., 1946, 61, 23.

2 Salle, A. J., and Jann, G. J., Proc. Soc. Exp. Biol. and Med., 1946, 62, 40.

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75	1	♂	37	75	10		,,
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15485

Relationship Between Body Temperature and Arterial Pressure.*

S. Rodbard and D. Feldman. (Introduced by L. N. Katz.)
From the Cardiovascular Department, Michael Reese Hospital, Chicago, Ill.

In the course of a series of investigations on the normal resting arterial blood pressures of various species of unanesthetized animals; data were obtained on the blood pressure of the turtle, *Pseudemys elegans*, at various body temperatures.

* Aided by the A. D. Nast Fund for Cardiovascular Research. The department is supported in part by the Michael Reese Research Foundation.

Arterial blood pressures were recorded on

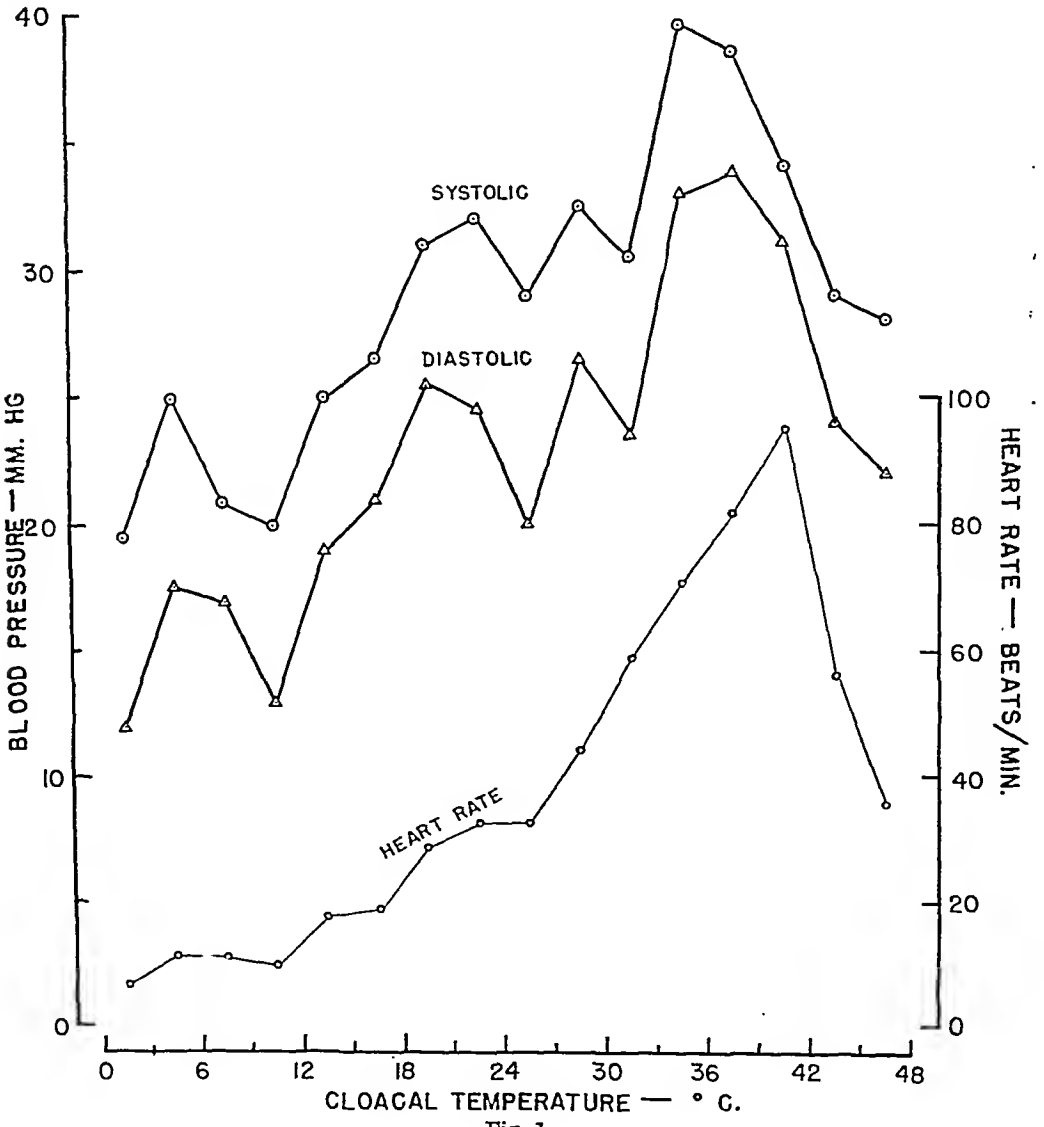

Fig. 1.

TABLE II.
Treatment of Anthrax in Guinea Pigs.

Date	Time	Animals inoculated intraperitoneally with 0.1 cc culture: Control not treated	Treated immediately cc subtilin	Treated after 3 hrs cc subtilin	Treated after 6 hrs cc subtilin	Treated after 9 hrs cc subtilin
3-5	2:00 PM	—	3			
	5:00 "	—	3	3		
	8:00 "	—	3	3	3	
	11:00 "	—	3	3	3	3
6	2:00 AM	—	3	3	3	3
	5:00 "	—	3	3	3	3
	8:00 "	—	3	3	3	3
	11:00 "	—	3	3	3	3
	2:00 PM	—	3	3	3	3
	5:00 "	—	3	3	3	3
	8:00 "	—	3	3	3	3
	11:00 "	—	3	3	3	3
7	2:00 AM	—	3	3	3	3
	5:00 "	—	3	3	3	3
	9:30 "	—	5	5	5	5
	1:00 PM	—	5	5	5	5
	10:00 "	—	5	5	5	5
8	10:00 AM	—	5	5	5	5
	4:00 PM	—	5	5	5	5
	7:00 "	Dead	5	5	5	5
9	9:00 AM		5	5	5	5
	5:00 PM		5	5	5	5
10	9:30 AM		4	4	4	4
	4:30 PM		5	5	5	5
	9:30 "		5	5	5	5
11	11:00 AM		5	5	5	5
	10:30 PM		5	5	5	5
12	10:30 AM		5	5	5	5
	9:30 PM		5	5	5	5
13	9:30 "		5	5	5	5
15	3:00 "		5	5	5	5
Results		Dead	Living	Living	Living	Living

before treatments were started. The 1st animal was treated immediately after being injected with the organism; the 2nd animal was given the first treatment 3 hours later; the 3rd animal was treated 6 hours later; and the 4th animal was treated 9 hours later. The subtilin preparation used was the same as in the first experiment. All treatments were given intraperitoneally. The schedule of treatments and results obtained are given in Table II.

It may be seen that the control guinea pig died in 77 hours whereas those treated with subtilin were still living after the 10th day, when the experiment was discontinued. The guinea pig treated as late as 9 hours after being inoculated with *Bacillus anthracis* was protected from the disease. The results indicate that subtilin exerts a powerful *in vivo* action on experimental anthrax infections in guinea pigs. The antibiotic did not produce any observable toxic reaction in the animals.

Conclusions. Subtilin has been shown to produce a powerful action on the course of experimental anthrax infections in guinea pigs. Animals treated with subtilin 9 hours after being injected with the organism were protected from the infection. The antibiotic exhibited no apparent toxic reaction in the animals.

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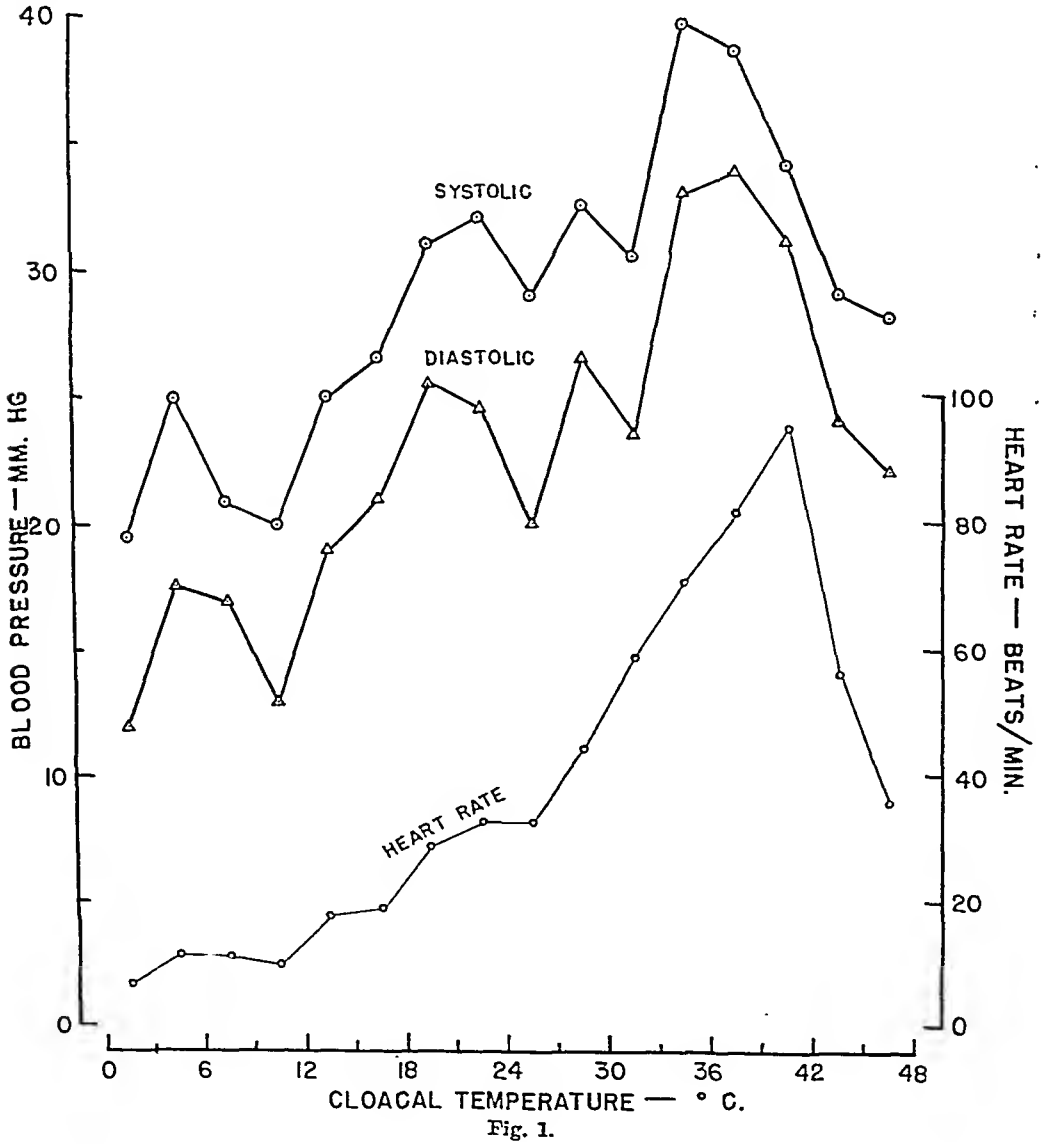

Fig. 1.

photographic paper, using the Hamilton optical recording manometer. A cannula attached to the manometer was directed cardially and tied into the left aortic arch. Body temperature was determined by means of a mercury thermometer tied into the cloaca. The animal was placed on its back in a pan of water, and the temperature of the water was changed slowly by the addition of ice cubes or by slow warming. The turtles were warmed from room temperature, 18°C, to about 40°, then cooled to about 3° and finally rewarmed to 45°. The rate of temperature change was about 0.3° per minute.

Arithmetic averages of the blood pressures and heart rates obtained in 2 heatings and one cooling of 5 turtles are given in Fig. 1. Our results show that there is a fairly consistent rise in arterial blood pressure as the cold animal is warmed until a temperature of about 38°C is obtained and with further warming the blood pressure falls rapidly. When the animals are then cooled the blood pressure increases until a temperature of about 38°C is obtained and with further cooling the blood pressure falls, so that in general the original curve is reduplicated. A second reheating gives data similar to that obtained in the first warming. The effect of temperature on heart rate is similar to that obtained with blood pressure, except that the heart rate is maximal at about 40°.

The pulse pressure remained relatively unchanged during our experiment. It decreased somewhat between 38° and 40°C when the heart rate was maximal, as can be seen in Fig. 1. It increased in 3 instances between 20° and 30°C in consequence of an unexpected fall of the diastolic pressure to zero.

Discussion. The rise in blood pressure seen with increasing body temperature may be related in some as yet undetermined way with the increased metabolic activity of the tissues. It is probably associated with an increase in cardiac output.

Comparison of our data on turtles with results on the resting blood pressure of unanesthetized mammals and birds, leads to the suggestion that there may be a general relationship between the body temperature and the level of the resting diastolic pressure. For example, the mammals (dog, rabbit, rat and man) which we have studied have all exhibited a basal diastolic arterial pressure of approximately 80 mm Hg.[1,2] These findings are in accord with scattered data found in the literature. Chickens, which have a normal body temperature of about 41°C, have a resting diastolic pressure of approximately 120 mm Hg.[3] Our data also appear to fit well with the relationship between blood pressure and body temperature in the new born rat, which has not yet attained the full development of the mechanism which assures homoiothermy.[4]

Summary. The arterial pressure of the turtle was found to vary directly with changes in body temperature between 3° and 38°C. Above 38°C the pressure declined.

1 Katz, L. N., Friedman, M., Rodbard, S., and Weinstein, W., *Am. Heart J.*, 1939, **17**, 334.

2 Rodbard, S., *Am. J. Physiol.*, 1940, **129**, 358.

3 Unpublished data.

4 Helmholz, H. F., Jr., *Fed. Proc.*, 1946, **5**, 44.

15486

Pressures Required to Produce Intradermal Wheals in Normal Human Subjects.

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A search of the literature has not disclosed any previous attempts to measure the range of pressure required to produce intradermal wheals in the normal human skin. Such pressures should be a direct measurement of the force necessary to spread the closely-bound

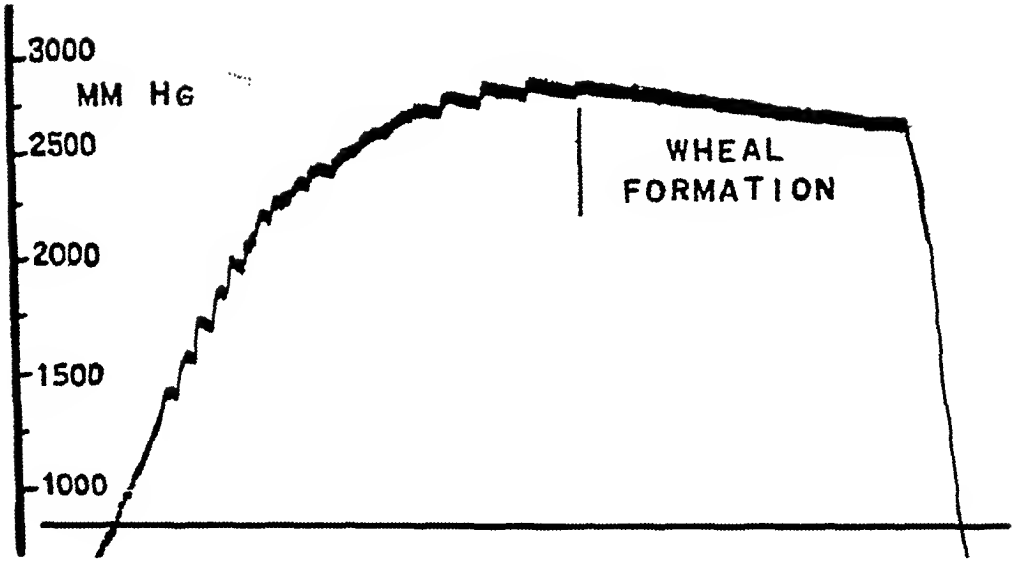

Fig. 1.

layers of the collagen connective tissue of the skin. Since natural changes may occur with varying degrees of hydration of the skin, the constancy of these pressures may be of clinical interest in such altered states as myxoedema and dehydration.

It was soon found that the pressure greatly exceeded that which could be measured by the usual Hg manometer and therefore the pressures were recorded optically. A glass spoon manometer was used with a calibration of 50 mm Hg pressure equal to 1 mm movement in the light beam which was recorded at 2½ meters on light-sensitive paper moving at a constant rate of 12 mm/second. The manometer was calibrated to 2750 mm of Hg by means of a mercury column. Wheals were raised on the lower half of the volar surface of the forearm, using a tuberculin syringe connected through a 3-way joint to the manometer and to a 27-gauge hypodermic needle ¼ inch in length. The pressure in this system was increased at a fairly rapid rate (approximately 750 mm Hg/second) until a wheal started to form. This position of the syringe plunger was then held until the wheal reached a diameter of 5 to 7 mm. To compare day-to-day variation, 5 measurements were made serially on each subject, and the average of these taken as the subject's wheal pressure for that day. Six male and 5 female subjects were used in this series.

Fig. 1 shows one of the typical pressure curves obtained. Even with this manometer it was necessary to set the zero level off the record in order to record on 60 mm paper the range of pressures involved. It should be pointed out that though we have not analyzed this part of the curve to date, the slope of the curve during wheal formation may be significant as a measure of cohesiveness of the collagen fibres of the skin.

The pressures obtained by this procedure are summarized in Table I. Considerable variation was found in consecutive readings. The average mean deviation of pressures made on the same subject on one day was 170 mm Hg with the bevel up, and 205 mm Hg with the bevel down. This is undoubtedly due to slight variations in the depth of the needle tip. Probably because of the inelasticity of the surface layers, measurements made with the bevel of the needle inserted up toward the epidermis were consistently higher than those made with the bevel down. Thus, the mean deviations on all subjects in each series and the large range of pressures in each case are not relatively as great as might be indicated by the individual figures. No consistent differences were found between

TABLE I.
The Pressures Required to Produce Intradermal Wheals.

No. of determinations	Characteristics of subjects: No.	Sex	Bevel of Needle	Solution	Pressures—mm Hg: Range	Mean pressure	Mean deviation
16	2	M	Up	Saline	1600-2800	2170	318
62	5*	M	Down	"	1000-3200	1970	454
14	3	F	Up	"	1900-3080	2240	128
24	5	F	Down	"	1280-2840	2160	400
44	4	M	"	Histamine acid phosphate 1-100,000 in saline	1260-3200	2460	405

* One Negro and one Japanese.

male and female skins, or among the skins of the Whites, Negroes, and Japanese studied. No attempt has been made in this preliminary study to determine the physiological variations in wheal-pressure which might occur with marked changes in environmental temperature or in the normal phases of the menstrual cycle. As shown in the table, the wheal-producing action of histamine is apparently too slow to lower the pressure required to produce wheals in this fashion. In fact the figures would rather indicate that histamine increased the pressure required.

Conclusion. The pressures required to produce intradermal wheals in normal individuals varied from 1,000 to 3,200 mm Hg with a mean pressure of about 2,000 mm Hg. No consistent differences in wheal pressures were found between males and females, or among Whites, Negroes and Japanese.

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Curative Action of Drugs in Lophurae Malaria of the Duck.*

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Scanty data have been published on the curative action of drugs in avian malarias, either in sporozoite or blood-induced infections.† No data has been published on the curative effect of drugs on lophurae malaria in the duck. For this reason, it appears advisable to record the following observations, despite the fact that the experiments are not perfectly controlled due to a large number of "accidental" deaths among our birds.‡

Infection was produced in ducks about 10 to 14 days of age by intravenous injection of 50×10^6 parasitized erythrocytes. The drug-diet method[1] of treatment was used. Administration of the diet was started 18 hours before infection and was continued for various lengths of time after infection. All drugs except SN 11,437, SN 187 and SN 475 were administered in the form of a salt.

* This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

† A considerable amount of data has been accumulated for various avian infections in the cooperative program of malarial research sponsored during the past five years by the Committee for Medical Research of the OSRD.

‡ These "accidental" deaths appear to be due to a filtrable agent. A preliminary study of this agent is reported elsewhere.[4]

[4] Dearborn, E. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 48.

[1] Marshall, Litchfield, and White, *J. Pharm. and Exp. Therap.*, 1942, 75, 89.

TABLE I.
Curative Action of Drugs in Lophuræ Malaria in the Duck.

Survey No.*		Duration of treatment days	Dose mg/kg/day	No. of ducks Used	Not Cured	"Cured"	Died†
359	Quinine (base)	6	180	10	9	0	1
		10	225	9	3	1	5
390	Quinacrine, dihydrochloride	6	280-310	12	6	0	6
		13	115	9	3	0	6
971	Pamaquine (base)	6	3.5	19	6	5	8
		6	9	44	16	17	11
		10	4	10	3	1	6
7618	Chloroquine, diphosphate	6	303	5	3	0	2
		6	183	8	5	0	3
11437	N¹-(5-chloro-2-pyrimidyl)-metanilamide	6	785	8	4	0	4
		26	225	7	5	0	2

* This number (SN) is used to designate a drug in the Monograph referred to above.[3]
† Figures in this column represent ducks which exhibited no evidence of infection but which died before "cure" could be established.

Subinoculations were done by intravenous injection of 2 cc of blood from the treated bird into a normal duck of about 150 g weight. Blood smears of the treated birds were made at the end of treatment, at the time of each subinoculation and as frequently as possible during the intervening periods. It was found that the injection of one to 10 parasitized erythrocytes into ducks of about 150 g weight produced an infection with the appearance of parasites in smear on the 10th-13th day. In some cases reinfection was attempted by injection of 20-30 $\times$ 10^8 parasitized erythrocytes into the treated ducks which at this time weighed about 800-1000 g. This dose in a normal duck of corresponding size would give a parasitemia of 40-50% erythrocytes parasitized on the 3rd day. The reinfected ducks were examined on the 3rd day. Those having 40% or more of erythrocytes parasitized were considered to be successfully reinoculated while those with less than 20% erythrocytes parasitized were considered to exhibit some degree of immunity.

The experiments given in this report are not as satisfactory as might be desired on account of the failure of either all the treated or all the subinoculated ducks to survive the necessary periods of observation. A series of experiments on uninfected untreated birds has shown that these deaths are not attributable to conditions of holding or procedures relevant to obtaining smears or subinoculating blood but to the filtrable contaminant mentioned in the note above. Obviously, the large number of deaths occurring before the observations are complete complicates the interpretation of the data; however, certain conclusions appear to be justified. As a result of deficiencies in our knowledge of the natural history of malaria in the duck, it must be recognized that while the presence of parasites in the blood of treated or subinoculated ducks is definitive evidence of failure to cure, inability to demonstrate either parasites in the blood or lack of immunity to them can only indicate probability of cure.

In Table I is given a very brief summary of the results obtained with the well known drugs, quinine, quinacrine and pamaquine, and also with chloroquine[2] and SN 11,437. This drug (SN 11,437) is rather unique in that it is a complete causal prophylactic in gallinaceum malaria in the chick, lophurae malaria in the turkey and cathemerium malaria in the canary.[3] However, it has no

[2] Board for the Coordination of Malarial Studies, *J. A. M. A.*, 1946, 130, 1069.

[3] A Survey of Antimalarial Drugs, 1941-45, sponsored by the Committee on Medical Research of the Office of Scientific Research and Development (Ed. F. Y. Wiselogle).

curative effect in a blood-induced infection of lophurae malaria in the duck. Quinine, quinacrine and chloroquine are not curative, while pamaquine appears to cure a fair percentage of ducks. When treatment is started 24 hours after infection, pamaquine is still curative. However, when treatment is started 48 hours after infection a much smaller per cent of cures is produced. In the table the criterion of "cure" is interpreted as 2 negative subinoculations, the last taken at least 26 days after infection. In our experience a small percentage of ducks having 2 negative subinoculations will prove to be infected if further subinoculations are done; however, this percentage is so small that it would not significantly change these data.

In addition to the drugs listed in Table I, we have found that SN 6911, 7-chloro-4-(4-diethylamino-1-methylbutylamino) - 3-methyl quinoline; SN 1796, α-(diamylaminomethyl)-1,2,3,4 - tetrahydro-9-phenanthrene-methanol; SN 187, 3',5'-dibromosulfanilanilide; SN 6520, 2 - (dimethylaminomethyl)-1-naphthol; SN 901, 6-chloro-2-methoxy-9-[3-(6-methoxy-8-quinolylamino)-propylamino] acridine; SN 475, 2,2',3,3'-tetramethyl-1,1'-diphenyl-4,4'-bi-3-pyrazoline-5,5'-dione; SN 11,426, 5-(*p*-chlorophenyl) - 1-isopropyl-1-methylbiguanide, salt with acetic acid; are not curative when given in maximal tolerated dosage for 6 days. However, SN 5241, α-(dinonylaminomethyl)-1,2,3,4-tetrahydro-9-phenanthrene methanol appears to cure a certain per cent of ducks when given in the maximal tolerated dosage. With the exception of SN 5241, pamaquine is the only drug examined which appears to cure lophurae malaria in a moderate percentage of ducks.

Summary. A number of drugs have been examined for their curative action in lophurae malaria in the duck. Pamaquine appears to cure a fair percentage of the birds, while quinine, quinacrine, chloroquine and a number of other drugs do not.

We wish to thank Charlotte Kennedy, Jean Hunt, Lucille Van Ghyl, and Evelyn Epperson for technical assistance.

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Filtrable Agents Lethal for Ducks.*

EARL H. DEARBORN. (Introduced by E. K. Marshall, Jr.)

From the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University.

In studying the curative activity of drugs against lophurae malaria in ducks it was found that approximately half the birds died with no demonstrable malaria before it could be established that they were cured. Extensive studies indicated that the deaths were not due to living conditions or to the various experimental procedures to which the birds were subjected. It appeared that the causative agent was in the blood injected to produce the malarial infection. Injection of blood from normal ducks did not produce deaths.

It seemed unlikely that the lethal agent was a toxin since the deaths were delayed 15 to 30 days after infection and since the causative agent was transferable by blood passage. It also seemed unlikely that it was a form of malaria since the birds which died had no detectable erythrocytic forms of the parasite and since studies in this and other laboratories[1] had failed to reveal any large number of exoerythrocytic forms of *P. lophurae* in the duck. Bacteriologic studies indicated that none of the common bacteria was responsible for the deaths. In view of these data it seemed likely that the lethal agent might be a filtrable organism. Subse-

* This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University.

[1] Porter, R. J., personal communication.

quent experiments showed that if plasma from ducks with lophurae malaria were filtered through a Seitz filter impervious to *Serratia marcessans* the filtrate produced no growth in broth at 38°C but was lethal for ducks when given intravenously.

This filtrable agent associated with *P. lophurae* caused the deaths of about 50% of the birds in 12 to 30 days. Twelve to 72 hours prior to death the birds became lethargic, anorexic and weak. These symptoms became progressively more severe and terminated in convulsions followed by death. This agent was serially passed 5 times by blood transfer. In addition, it was carried with the lophurae malaria through biweekly blood transfer for at least 2 years. Passage of the malarial parasite through mosquitoes, chicks, canaries or turkeys† failed to eliminate the filtrable agent. Washing of the parasitized erythrocytes with normal duck plasma or glucose-Ringer's solution, hemolysis with 0.05% saponin and rewashing of the parasite-containing residue with normal plasma or glucose-Ringer's solution failed to eliminate the filtrable agent. This lethal effect was also associated with *P. lophurae* in ducks obtained from other laboratories. Various attempts to obtain malaria parasites, free of the filtrable agent, from ducks which had survived an infection with both also failed. A number of drugs which were tried for their curative effect in malaria had no detectable effect on the filtrable agent. Decreasing the dose of this agent decreased the percent of ducks which die. This agent was not lethal to white mice when given intravenously, intraperitoneally or intracerebrally, and it was not lethal to dogs when given in large doses intravenously.

A lethal filtrate was obtained by filtration of plasma from ducks infected with *P. cathemerium*. It was serially transferred 35 times by blood inoculation. When first obtained this agent was 100% fatal in 3 to 5 days. Its lethality was unaffected by sulfaguanidine, penicillin, sulfadiazine and a number of potent antimalarial drugs. This rapidly lethal strain was lost; however, the agent was obtained again but it was only lethal in 10 to 20 days. Various attempts to increase its virulence failed. This agent could not be washed from the parasitized erythrocytes either before or after hemolysis. Administration of the filtrate by various routes had no effect on the time of death. Apparently ducks which had survived infection with both *P. cathemerium* and this filtrable agent possessed some degree of immunity to reinfection with the filtrable agent.

Filtrates which were lethal for ducks were also obtained by Seitz filtration of plasma from ducks infected with *P. relictum* or *P. elongatum*. The former was lethal in 30 to 40 days while the latter required only 3 to 6 days. The agent associated with elongatum malaria was transferred serially 5 times by blood passage. A quantity of pooled plasma from several birds infected with this filtrate was stored at the temperature of solid carbon dioxide.

Summary. Filtrable agents, lethal for ducks, were obtained by Seitz filtration of plasma from ducks with lophurae, cathemerium, relictum or elongatum malaria. Attempts to free the malaria of the filtrable agent were unsuccessful.

† We wish to thank Dr. R. J. Porter of the University of Michigan for passing the parasites through mosquitoes, canaries, and turkeys.

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Immunological Similarity of Streptococcal Antifibrinolysins.*

MELVIN H. KAPLAN AND THE COMMISSION ON ACUTE RESPIRATORY DISEASES.†
From the Respiratory Diseases Commission Laboratory, Regional Station Hospital, Section 2, Fort Bragg, North Carolina.

The development of antifibrinolytic properties in the blood of patients convalescing from streptococcal disease is commonly regarded as an immunological response to infection by the β-hemolytic streptococcus.[1-4] Measurement of the antifibrinolytic capacity of the blood has consequently been employed as a diagnostic aid in the study of infections due to this organism.[2-7]

In general, the capacity of the blood to resist the action of fibrinolysin is measured by the time required for the plasma clot to undergo complete dissolution, such tests being performed either on the patient's plasma clot[1] or on a normal plasma clot to which serum of the patient has been added.[8,9] From the results of studies with fibrinolysins from 40 different strains, Van Deventer[10] suggested that they all probably belong to one immunological type. In contrast, Mote, Massell and Jones,[11] using sharper quantitative methods, found that the resistance of plasma clots varied with fibrinolysins derived from different strains. They attributed their results to immunological differences in antifibrinolysins; the nature of these immunological differences, however, was not investigated further.

Recent investigation has indicated that antifibrinolysin is not the only factor in the blood which may confer antifibrinolytic properties on a plasma clot. Studies of the mechanism of the fibrinolytic reaction have shown that the actual lytic agent in streptococcal fibrinolysis is not fibrinolysin, but a proteolytic enzyme in the plasma (lytic factor) which is activated by fibrinolysin.[12,13] Accordingly, antifibrinolytic effects may result not only from the presence in the plasma of antifibrinolysin, but also from increased amounts of antiprotease.[14,15] In addition, it

* This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation, and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

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13 Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

14 Mirsky, I. A., *Science*, 1944, **100**, 198.

15 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 337.

TABLE I.

Determination of Antifibrinolysin Titers in Convalescent Phase Sera by Means of Fibrinolysins from Groups A, C, and G Streptococci.

Group of infecting organism	Convalescent serum No.	Titer with Group A fibrinolysin	Titer with Group C fibrinolysin	Titer with Group G fibrinolyhin
A	100 B	83	83	83
	144 B	625	625	625
	167 B	278	278	278
	234 B	125	179	179
	463 B	179	179	179
C	120 C	83	83	56
	249 B	125	125	125
	258 C	56	56	56
	581 B	<25	25	25
	1070 C	<25	<25	<25
G	8 C	<25	<25	<25
	549 C	25	25	25
	6 C	<25	25	25
	971 D	56	56	83
	694 F	36	56	36

has been suggested that clot resistance may be due, in some instances, to a deficiency of the lytic factor in the plasma.[16,8]

Since the effect of these nonspecific antifibrinolytic factors was not controlled in previous work, the problem of the serological specificity of antifibrinolysins was reinvestigated. Antifibrinolysin was measured quantitatively by a serological method which minimized the effect of antiprotease and deficiency of lytic factor. The sera studied were obtained from patients infected by streptococci from 3 different Lancefield groups: A, C and G. In order to test for possible differences in antifibrinolysins, these sera were titrated with fibrinolysins derived from strains from each of these 3 different groups, and the resulting titers compared. The results of these studies showed that the antifibrinolysins tested in this manner were immunologically similar.

Methods. The sera tested were obtained from patients with acute streptococcal pharyngitis or tonsillitis. Streptococcal etiology was confirmed in each case by the presence of β-hemolytic streptococci in 2 or more cultures of the throat, and by the development of antistreptolysin "O" antibodies during early convalescence.[17] Streptococci were grouped by the Lancefield precipitin-tube technic[18] using bacterial extracts prepared by Fuller's method.[19] An infection was attributed to a strain of a given group if all of the throat cultures taken during the acute illness showed other groups to be entirely absent.

Antifibrinolysin levels were determined by the procedure previously described.[20] The sera were first diluted serially in 1.5-fold dilutions, beginning with a 1/25 dilution, as follows: 1/25, 1/36, 1/56, 1/83, 1/125, 1/179, 1/278, 1/417, 1/625, 1/900, 1/1400. Each dilution was incubated with a standard amount of fibrinolysin. The titer of a given serum was the highest dilution which neutralized the lysis-promoting activity of the standard unit of fibrinolysin. A difference in titer of 2 dilution increments (2 tubes), corresponding to a 2.25-fold antibody change, was considered to be beyond the error of the method.

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14 Mirsky, I. A., *Science*, 1944, **100**, 198.

15 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 337.

However, a second explanation was suggested by the report[11] that antifibrinolysins differed immunologically. If antibody differences were a significant factor in the determination of antifibrinolysin, it seemed possible that the detection of antibody rises might be limited to the variety of fibrinolysin employed as antigen. The present study was consequently undertaken to test for and, if possible, to determine the nature of the differences in antifibrinolysins.

An attempt was made to obtain evidence of such differences by measuring the antibody level of the sera of patients infected by streptococci belonging to different Lancefield groups. Quantitative antibody titrations were carried out on the sera of patients with infections due to organisms from each of the 3 groups, A, C and G; and the fibrinolysins used for testing for antibody were derived from strains from each of these same groups. The results of the study showed that the titers of the various sera tested were essentially the same with all 3 fibrinolysins.

It would thus appear that the fibrinolysins produced by these different organisms were immunologically identical in their reactivity. This is in agreement with the observations of other workers.[3,10,26] It is not possible to explain the different results reported in the first quantitative studies of the problem.[11] However, as possibly contributing to apparent differences, 2 points may be mentioned: (1) the nonspecific factors in the plasma, such as antiprotease or a deficient lytic factor, may have produced the differences observed, or (2) the variations may have been due to the method of antibody estimation employed. Measurement of antibody was based on the amount of fibrinolysin which permitted clot dissolution. Since fibrinolysin and antifibrinolysin combine in varying multiple proportions,[15] it would appear that a procedure in which the fibrinolysin concentration is varied is not suitable for the measurement of antibody. The evidence thus suggests that the fibrinolysins produced by streptococci pathogenic for man are identical in their immunological behavior.

Conclusion. It was concluded, therefore, that the use of a single fibrinolysin preparation is satisfactory for the serological determination of antifibrinolysin in human infections.

[26] Kirby, W. M., and Rantz, L. A., *Arch. Int. Med.*, 1943, **71**, 620.

15490

Action of Estrogen on Release of Hypophyseal Luteinizing Hormone.

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The gonad stimulating properties of the pituitary are better understood than is the reciprocal action of the gonadal secretions on the pituitary. In respect to the latter process the terms "suppression" and "inhibition" are frequently used to describe what is obviously a more complicated mechanism than these empirical terms imply. The pituitary gonadotropic complex does not always act as a unit and individual factors should be considered separately in their response to estrogens and androgens. Furthermore, the intracellular synthesis of a pituitary factor is a process distinct from that involving the liberation of the factor from the gland.

We have attempted in the work presented here and in our previous studies,[1,2] to obtain

[1] Hellbaum, A. A., and Greep, R. O., *Am. J. Anat.*, 1940, **67**, 287.

[2] Hellbaum, A. A., and Greep, R. O., *Endocrinology*, 1943, **32**, 33.

TABLE II.
Determination of Antifibrinolysin Titers of Acute and Convalescent Phase Sera by Means of Fibrinolysins of Homologous and Heterologous Groups.

Group of infecting organism	Case No.	Date of serum	Antistreptolysin "O" titer	Titer with Group A fibrinolysin	Titer with Group C fibrinolysin	Titer with Group G fibrinolysin
A	O-463	1-1	159	56	56	56
		2-27	400	179	179	179
A	O-144	12-15	159	125	125	125
		1-12	317	625	625	625
A	O-167	12-21	159	83	125	125
		1-19	500	278	278	278
C	T-101	5-20	50	<25	<25	<25
		6-11	159	36	36	36
G	O-6	10-21	125	<25	<25	<25
		1-26	400	25	25	25
G	O-694	1-1	62.5	25	36	36
		2-23	100	36	56	56

The *fibrinolysins* were prepared from culture filtrates by alcoholic precipitation according to the procedure of Garner and Tillett,[21] and were stored in the frozen state until ready for use.

Results. The titers of each of 15 convalescent sera were determined with fibrinolysins derived from strains belonging to the 3 groups, A, C and G. Five of these 15 sera were obtained from patients convalescent from a group A infection; 5 were from patients with a group C infection; and the 5 remaining were from cases infected by group G strains.‡ As shown in Table I, the titer of each serum was essentially the same with each of the 3 fibrinolysins employed. Variation of the titer of any one serum did not exceed one tube. The evidence thus indicated that the 3 fibrinolysins reacted with the sera in a quantitatively identical manner.

In a second experiment these 3 different fibrinolysins were used to measure the increase in antifibrinolysin in serum specimens collected during the acute and convalescent phases of proved streptococcal infections. Sera were examined from 3 patients with group A infections, one patient with a group C infection, and 2 patients with group G infections.

As shown in Table II, the antibody rises in the group A and C cases were at least 2 tubes in magnitude; the group G cases showed increases of one tube. The amount of antibody increase in each of these patients was essentially the same with all 3 fibrinolysins employed. It was concluded that the antifibrinolysins produced in human infections due to groups A, C and G streptococci were immunologically similar.

Discussion and Summary. Several studies have indicated that a rise of antifibrinolysin apparently occurs much less frequently in streptococcal disease than a rise in antistreptolysin.[23,24,5,7] One possible explanation for this low frequency of the antifibrinolysin response is that streptococci vary markedly in their ability to produce fibrinolysin, and consequently in the frequency which they may stimulate an antibody response. Evidence has been presented in support of this hypothesis.[25,5]

21 Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

‡ The detailed clinical, bacteriological, and serological data on the patients with group C and G infections are to be published elsewhere.[22]

22 Commission on Acute Respiratory Diseases, *New Eng. J. Med.*, to be published.

23 Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1935, **16**, 513.

24 Winblad, S., *Acta Path. et Microbiol. Scand.*, 1941, Supp. 44, 1.

25 Commission on Acute Respiratory Diseases and Kaplan, M. H., *Science*, 1945, **101**, 120.

of the normal rat contains moderate amounts of the luteinizing hormone. There were from 2 to 5 corpora lutea in the ovaries of each of the recipients.

Spayed Adult Female Rats. In this group, 28 adult female rats were oophorectomized 3 months prior to necropsy and removal of the pituitary glands for assay. A total of 20 normal and 19 hypophysectomized recipients were used. The pituitaries from this donor group showed a marked increment in the luteinizing factor. The ovaries of the recipient rats were extensively luteinized. Macroscopic and histologic examination revealed ovaries filled with corpora lutea and few, or no maturing follicles.

In addition to an increase in the residual luteinizing factor, following spaying, the pituitary content of follicle-stimulating hormone was likewise increased. Less than 1/10 of the dose of pituitary powder from spayed rats was required to produce an ovarian weight increase comparable to that produced by the pituitaries of normal animals. This was undoubtedly due, in part, to an increased storage of the luteinizing hormone which acted synergistically with the follicle-stimulating hormone to produce greater ovarian enlargement.

Oophorectomized Adult Female Rats Injected with Estradiol Benzoate. The object of this experiment was to determine whether the store of luteinizing hormone, which had accumulated in the pituitary as a result of spaying, could be released by estrogen injection and be reduced to such a degree that its physiological activity would be undetectable in recipient female rats.

A total of 21 adult animals, which had been spayed 3 months previously, were injected daily with 15 μ estradiol benzoate* in oil for 30 to 45 days. The pituitaries of these animals were tested in 7 intact and 7 hypophysectomized recipients. The ovaries of only one of the assay animals contained corpora lutea. The ovaries of the remaining 13 animals showed only follicle stimulation. The presence of lutein tissue in the single intact recipient may have been due to endogenous luteinizing hormone from the test animal's own pituitary gland.

Another group of 9 adult oophorectomized rats were injected with 3 μ of estradiol benzoate daily for periods varying from 30 to 45 days. These pituitaries were administered to 9 hypophysectomized recipients. The ovaries of none of these animals showed a trace of luteinization, although most of them had well developed follicles.

It is clear from these data that estrogen caused a liberation of the luteinizing hormone to a point where this substance was being released as rapidly as it was being produced. This finding is in accord with our previously expressed views regarding the effect of the gonads on the pituitary. Although our former study[2] was concerned primarily with the action of testosterone, we doubt that there is an essential qualitative difference between the action of estrogen and testosterone on the pituitary with respect to their ability to cause a release of the luteinizing hormone. This view concurs with the findings of other workers investigating functional activity of the pituitary, with respect to gonad stimulation, during treatment with sex hormones.[3-7] Laqueur and Fluhmann[8] injected testosterone into normal female rats and obtained a diminution of gonadotropic potency (by implants) which they attributed to an inhibition of the production of luteinizing hormone.

Summary. The pituitaries of adult female rats, under normal estrogen influence, stimulated moderate luteinization in normal and hypophysectomized recipient immature female rats. The pituitaries of adult female rats freed of estrogen stimulation through oophorectomy, invariably caused extensive luteinization of the recipient ovaries. This

* The estradiol benzoate was kindly furnished by the Schering Corporation, Bloomfield, N.J.

[3] Hohlweg, W., *Klin. Wchnschr.*, 1934, **13**, 92.

[4] Selye, H., Collip, J. B., and Thomson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1377.

[5] Wolfe, J. M., and Hamilton, J. B., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 189.

[6] Fevold, H. L., Hisaw, F. L., and Greep, R. O., *Am. J. Physiol.*, 1935, **114**, 508.

[7] Freed, S. C., Greenhill, J. P., and Soskins, S., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 440.

[8] Laqueur, G. L., and Fluhmann, C. F., *Endocrinology*, 1942, **31**, 300.

TABLE I.

Comparison of the Residual Pituitary Gonadotropic Activity Found in: Normal Adult Female Rats; Female Rats 3 Months After Spaying; Spayed Female Rats Treated with Estrogen for 30 to 45 Days Beginning at 3 Months After Spaying.

Adult female donors			Immature recipient female rats			
				Ovarian wt		
Type	No. of animals	Avg dose (mg)	No. of animals	Avg (mg)	Range (mg)	Type of ovarian response
Normal 21-day-old female recipients.						
Intact	38	14.5	7	29.1	16- 49	2 to 5 corpora lutea
Spayed 3 months	8	3.6	8	73.9	32-135	Numerous corpora lutea
	6	1.7	12	39.0	23- 54	" " "
Spayed 3 months then treated with 15μ estradiol benzoate 30 to 45 days	14	13.8	7	31.3	20- 42	6 rats with follicles only 1 rat with corpora lutea
Avg ovarian wet of uninj. immature control rats—12 mg.						
Hypophysectomized Female Recipients						
Intact	40	19.2	5	27.2	21- 34	2 to 5 corpora lutea
Spayed 3 mo	9	3.54	9	54.8	34-103	Numerous corpora lutea
	5	1.7	10	32.4	19- 56	" " "
Spayed 3 mo then treated with 15μ estradiol benzoate 30 days	7	6.4	7	20.2	16- 28	Follicles only
Spayed 3 mo then treated with 3μ estradiol benzoate 30-45 days	9	5.8	9	15.4	9- 26	" "
Avg ovarian wgt of 8 hypophysectomized immature controls—8.7 mg.						

information on the storage and release of pituitary gonadotropins. The purpose of this report is to demonstrate the qualitative changes in the residual luteinizing component of the pituitary at various levels of estrogen influence.

The experimental procedure consisted of comparing the type of ovarian stimulation produced by the pituitaries of: (1) normal adult female rats; (2) adult female rats 3 months after the removal of the ovaries; (3) oophorectomized adult female rats injected with estradiol benzoate after a 3-month postoperative interval. The pituitaries of the different donor groups were assayed in normal female rats 21 days of age and in immature hypophysectomized female recipients.

The donor pituitaries, following removal, were dehydrated in acetone, dried, and powdered by the procedure previously reported.[1] The powder was suspended in water and injected twice daily for 3 days into normal and hypophysectomized recipients. The latter received their first injection 48 hours after the operation. Necropsies were made 120 hours after the first injection. The ovaries were weighed and examined before fixation for follicle growth and for presence of corpora lutea. Histological sections were prepared when corpora lutea were not grossly visible. The data are summarized in Table I.

Normal Adult Female Rats. Assays carried out on the pituitaries of this group revealed the characteristic gonad stimulating activity of pituitaries which have been subjected to the normal output of ovarian hormones. A total of 78 glands were assayed in 7 normal and 5 hypophysectomized recipients (Table I). The resultant ovarian responses showed that the pituitary gland

of the normal rat contains moderate amounts of the luteinizing hormone. There were from 2 to 5 corpora lutea in the ovaries of each of the recipients.

Spayed Adult Female Rats. In this group, 28 adult female rats were oophorectomized 3 months prior to necropsy and removal of the pituitary glands for assay. A total of 20 normal and 19 hypophysectomized recipients were used. The pituitaries from this donor group showed a marked increment in the luteinizing factor. The ovaries of the recipient rats were extensively luteinized. Macroscopic and histologic examination revealed ovaries filled with corpora lutea and few, or no maturing follicles.

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Another group of 9 adult oopborectomized rats were injected with 3 μ of estradiol benzoate daily for periods varying from 30 to 45 days. These pituitaries were administered to 9 hypophysectomized recipients. The ovaries of none of these animals showed a trace of luteinization, although most of them had well developed follicles.

It is clear from these data that estrogen caused a liberation of the luteinizing hormone to a point where this substance was being released as rapidly as it was being produced. This finding is in accord with our previously expressed views regarding the effect of the gonads on the pituitary. Although our former study[2] was concerned primarily with the action of testosterone, we doubt that there is an essential qualitative difference between the action of estrogen and testosterone on the pituitary with respect to their ability to cause a release of the luteinizing hormone. This view concurs with the findings of other workers investigating functional activity of the pituitary, with respect to gonad stimulation, during treatment with sex hormones.[3-7] Laqueur and Fluhmann[8] injected testosterone into normal female rats and obtained a diminution of gonadotropic potency (by implants) which they attributed to an inhibition of the production of luteinizing hormone.

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* The estradiol benzoate was kindly furnished by the Schering Corporation, Bloomfield, N.J.

[3] Hohlweg, W., *Klin. Wchnschr.*, 1934, **13**, 92.

[4] Selye, H., Collip, J. B., and Thomson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1377.

[5] Wolfe, J. M., and Hamilton, J. B., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 189.

[6] Fevold, H. L., Hisaw, F. L., and Greep, R. O., *Am. J. Physiol.*, 1935, **114**, 508.

[7] Freed, S. C., Greenhill, J. P., and Soskins, S., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 440.

[8] Laqueur, G. L., and Fluhmann, C. F., *Endocrinology*, 1942, **31**, 300.

indicated that liberation of the hypophyseal luteinizing factor was minimal in the absence of estrogen; the factor remaining stored within the pituitary gland.

Release of the luteinizing factor by estrogen was suggested also by the type of ovarian response produced by pituitaries from oophorectomized adult female rats which had been injected with estradiol benzoate daily for 30 to 45 days. These pituitaries produced follicular development but no corpora lutea, due to removal of the luteinizing factor by the estrogen treatment.

15491 P

Effect of Long Chain Fatty Acids on Bacterial Growth.

R. J. DUBOS.

From the Laboratories of The Rockefeller Institute for Medical Research, New York City.

We have shown elsewhere that certain water soluble lipids promote diffuse growth of tubercle bacilli in synthetic media, especially in the presence of serum albumin.[1,2] These same substances have now been found to enhance the growth of other microbial species, in particular of an unidentified micrococcus (strain C), recently isolated in our laboratory. Some understanding has also been gained of the conditions under which long chain fatty acids can stimulate bacterial growth.

It is known that the soaps of fatty acids exert a bacteriostatic and bactericidal effect on certain micro-organisms, particularly on the Gram-positive and acid-fast species, and that unsaturated acids are more toxic than the corresponding saturated compounds.[3-9] For example, concentrations of oleic acid as low as 0.000001-0.00001% are sufficient to cause inhibition or retardation of growth of small inocula of human tubercle bacilli in synthetic liquid media. On the other hand, fatty acid esters (methyl oleate, triethanolamine oleate, phosphatides) exhibit little or no primary toxicity. That the lack of toxicity is not due to poor solubility of the esters is indicated by the fact that the polyoxyethylene derivatives of oleic acid are essentially nontoxic, even though they are completely dispersible in water.[1,2] Thus, tubercle bacilli grow readily in synthetic media to which has been added 0.1-1.0% of Tween 80 (a polyoxyethylene derivative of sorbitan monooleate) purified to remove unesterified fatty acid.[10] Detoxification of the fatty acids can also be achieved by adding to the medium native serum albumin. When an adequate amount of this protein is added to an opalescent soap emulsion (at neutral pH), there occurs an immediate clearing of the emulsion with concomitant disappearance of toxicity. It takes approximately 40 parts by weight of albumin to achieve complete detoxification of 1 part of oleic acid; however, growth of tubercle bacilli can be obtained in media containing 0.01% oleic acid and 0.5% serum albumin if a sufficiently large inoculum is used.

When rendered nontoxic, either by ester-

1 Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

2 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

3 Avery, O. T., *J. Am. Med. Assn.*, 1918, **71**, 2050.

4 Bergström, S., Theorell, H., and Davide, H., *Nature*, 1946, **157**, 306.

5 Boissevain, C., *Am. Rev. Tub.*, 1926, **13**, 84.

6 Hettche, H. O., and Weber, B., *Arch. Hyg. Bakt.*, 1940, **123**, 69; *Biochem. J.*, 1945, **39**, 78.

7 Kodicek, E., and Worden, A. N., *Nature*, 1946, **157**, 587.

8 Lamar, R. V., *J. Exp. Med.*, 1911, **13**, 1,380; **14**, 256.

9 Stanley, W. M., Coleman, C. H., Green, C. M., Sacks, J., and Adams, R., *J. Pharm. Exp. Ther.*, 1932, **45**, 121.

10 Davis, B. D., and Dubos, R. J., *Arch. Biochem.*, 1946.

TABLE I.
Effect of Oleic and Stearic Acid on Bacterial Growth.

Added to casein hydrolysate medium				Growth in mg/10 ml*—7 days incubation	
Glucose %	Oleic acid %	Stearic acid %	Albumin %	Micrococcus C mg	Avian tubercle bacillus mg
0	0	0	0	0.2	0.1
0	0.001	0	0	1.0	0
0	0.01	0	0	0	0
0.5	0	0	0	0.3	0.1
0.5	0.001	0	0	2.1	0
0.5	0.01	0	0	0	0
0	0	0	0.5	0	0.2
0	0.001	0	0.5	1.0	0.3
0	0.01	0	0.5	3.9	1.1
0.5	0	0	0.5	0	0.3
0.5	0.001	0	0.5	4.1	0.5
0.5	0.01	0	0.5	5.1	1.1
0	0	0.001	0.5	0	0.3
0	0	0.01	0.5	0	0.8
0.5	0	0.001	0.5	0	0.6
0.5	0	0.01	0.5	0	1.2

* The amount of growth was evaluated from measurements of optical density and of sediments obtained by centrifugation in comparison with suspensions containing known weights of bacteria.

ification or by admixture with serum albumin, a number of long chain fatty acids are found able to enhance the growth of certain bacteria; the different bacterial species, however, differ markedly in their response to the various acids. The comparative behavior of tubercle bacilli and of micrococcus C in this respect can be summarized in the following statements.

Enhancement of growth of tubercle bacilli can be obtained by adding 0.01% of any of a variety of long chain fatty acids—saturated or unsaturated—to a medium containing 0.5% crystalline serum albumin. The presence of glucose or of other readily available carbon compounds is not necessary for the development of growth or for demonstration of the enhancing effect of the fatty acid. This finding is in agreement with the fact that several long chain fatty acids have been found to stimulate oxygen uptake by tubercle bacilli.[11] In the case of micrococcus C, on the other hand, abundant growth results from the addition of oleic, linoleic, linolenic or aracbidonic acids (0.0001-0.001%) to a mineral medium containing glucose as the sole source of carbon. Saturated fatty acids, on the contrary, appear completely unable to permit growth. In this respect the nutritional requirements of micrococcus C are similar to those of diphtheria and tetanus bacilli.[12,13] Altbougb none of the other substances tested (yeast extract, hydrolysates of casein, protein, etc.) can take the place of the unsaturated fatty acids in initiating growth of the micrococcus, the abundance of the growth is markedly increased by the addition of glucose to a medium containing an adequate concentration of unsaturated acid. Finally, addition of crystalline albumin to media containing only minute concentrations of unsaturated acids completely inhibits the small amount of growth of micrococcus which would have taken place in the absence of the protein; this inhibitory effect can be neutralized by adequate addition

[11] Loebel, R. O., Shorr, E., Richardson, H. B., *J. Bact.*, 1933, **26**, 139.

[12] Cohen, S., Snyder, J. C., and Mneller, J. H., *J. Bact.*, 1941, **41**, 581.

[13] Feeney, R. E., Mueller, J. H., and Miller, P. A., *J. Bact.*, 1943, **46**, 559.

of oleic, linoleic, linolenic or arachidonic acid. No similar growth inhibitory effect by albumin has been observed in the case of tubercle bacilli.

These different phenomena are illustrated in Table I in which are compared the growths of a strain of avian tubercle bacilli and of micrococcus C in a casein hydrolysate medium to which oleic acid, stearic acid, glucose and serum albumin were added as indicated.

More extensive data concerning the effect of different fatty acids on bacteria—both in liquid and on agar media—will be presented in a forthcoming publication; it will be shown also that, at equal concentrations of long chain fatty acids, the water soluble esters are more efficient than the corresponding soaps in supporting bacterial growth.

It appears worth pointing out at this time that, under the proper cultural conditions, the amount of growth yielded by micrococcus C seems to be directly related to the amount of unsaturated fatty acids present in the medium (between 0.00001 and 0.0001%). This property suggests that the culture might lend itself to the development of a microbiological assay method for these lipids.

15492

Heart Rate of the Albino Rat.*

LOUIS E. MOSES. (Introduced by F. E. Emery.)

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Hoskins, Lee and Durrant[1] counted the heart rates of rats by stethoscope while the animals lay dozing in their cages, and they reported a basal rate of 281±18 beats per minute. Fishburne and Cunningham[2] wrapped their rats in a towel for auscultation of the heart sounds; the rates they obtained fell in the neighborhood of 300 beats per minute. Meyer and Yost[3] held their rats in the crook of the arm while palpating the apex beat, and they found the rates of adult rats to vary between 300 and 330 beats per minute.

Drury, Harris and Maudsley,[4] Robertson and Doyle,[5] and Leblond and Hoff[6] recorded electrocardiograms in rats and thus avoided subjective errors, but they did so while forcibly restraining their rats in the supine position to allow insertion of needle electrodes through the skin. Kniazuk[7] described an oscillographic method for obtaining electrocardiograms from rats placed in specially constructed cages. The method developed independently by the present author follows the same principle as that of Kniazuk but has the advantage of making inexpensive permanent records. It involves registration of the electrocardiogram from the surfaces of the rat's feet.

At first, the record was transcribed with the usual electrocardiograph, but, aside from the cost of bromide paper and the time lost in developing the film, that procedure was unsatisfactory in that it failed to tap the heart's potential through plantar callouses that the rats acquired in their mesh-floored cages.

The apparatus finally adopted was that developed for the recording of brain waves

* Publication No. 596, research series, the University of Arkansas.

1 Hoskins, R. G., Lee, M. O., and Durrant, E. P., *Am. J. Physiol.*, 1927, **82**, 621.

2 Fishburne, M., and Cunningham, B., *Endocrinology*, 1938, **22**, 122.

3 Meyer, A. E., and Yost, M., *Endocrinology*, 1939, **24**, 806.

4 Drury, A. N., Harris, L. J., and Maudsley, C., *Biochem. J.*, 1930, **24**, 1632.

5 Robertson, E. C., and Doyle, M. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 139.

6 Leblond, C. P., and Hoff, H. E., *Am. J. Physiol.*, 1944, **141**, 32.

7 Kniazuk, M., *J. Lob. Clin. Med.*, 1937, **22**, 868.

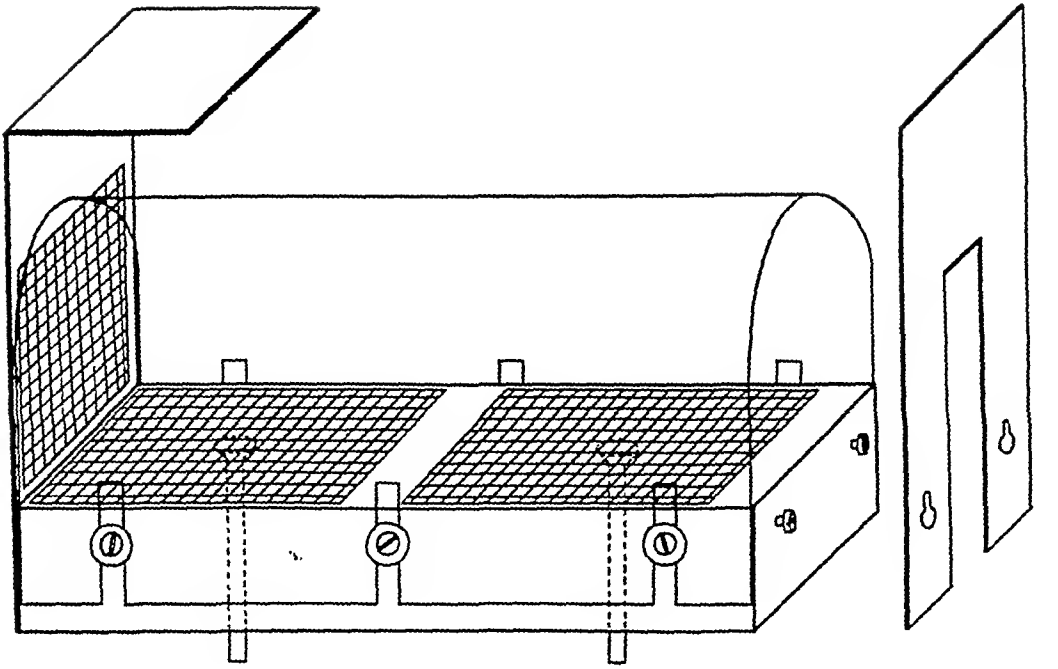

FIG. 1.

Rat holder for heart rate determination. Approximately one-fourth actual size. Description in text.

by Gerard and his associates. In this method, the impulse is picked up by contact electrodes in the form of copper floor plates, stepped up by Offner's[8] 3-stage push-pull amplifier, and inscribed on adding machine tape by the piezo-electric crystograph of Offner and Gerard.[9] Since the tape is moved by a constant speed motor, the heart rates can be measured accurately by the use of dividers.

Construction of the animal holder is shown in Fig. 1. The floor is a 1″ x 2½″ x 6″ hard rubber block into which copper plates are countersunk to act as the pick-up electrodes, one for both forefeet and the other for the hind feet. To simulate the rough floor to which rats are apparently partial, a square of copper screening is soldered to the surface of each of the copper plates. A long iron screw, soldered to each plate, passes through the hard rubber floor to dip into a mercury cup set into the shelf on which the cage rests. The potentials are led from these mercury cups into the amplifier and crystograph. Animal, cage, and wiring are shielded from 60-cycle hum by enclosure in a large copper screen chamber, appropriately grounded.

Neither electrode paste nor saline is required to facilitate transmission from the rat to the electrodes on which he is standing.

To make each cage adjustable for rats of all sizes, the sides and roof are formed by a single bent sheet of heavy celluloid, measuring 7″ x 6″. Slots cut into the celluloid sheet in place of screw holes thus make it possible to alter the volume of the cage at will.

The front gate is constructed of heavy sheet metal, 2¾″ x 6½″, with a large copper screen window for ventilation. The top of this front gate is bent to form a 2-inch projection to shade the animal's head. (This author has found no merit to the almost universal assumption that bright light induces rats to sleep; it probably irritates their eyes,

[8] Offner, F., *Rev. Sci. Instruments*, 1937, **8**, 20.

[9] Offner, F., and Gerard, R. W., *Science*, 1936, **84**, 209.

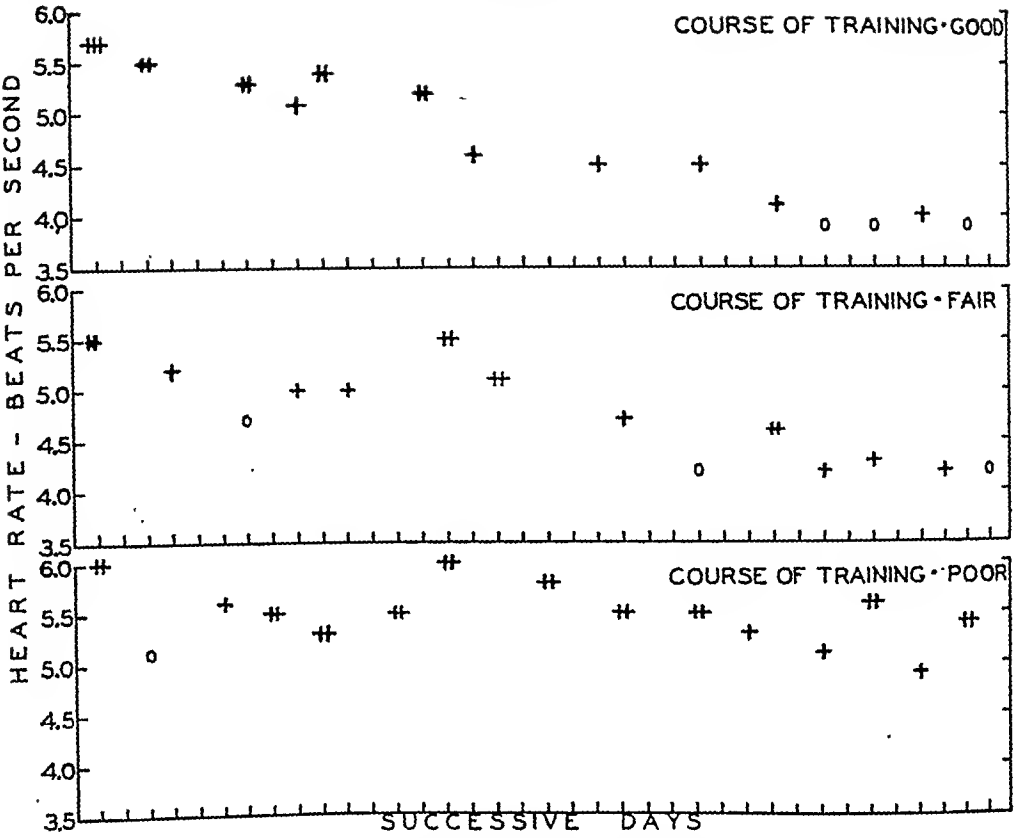

FIG. 2.

Decline in heart rate with progressive training. Evaluation of activity: ++++, extreme and prolonged; +++, intermittent but great; ++, moderate; +, occasional and mild; 0, insignificant; ∞, prolonged inactivity.

which are unprotected by pigment. Actually, it was observed that coating the shiny front screen with flat black paint aids significantly in producing relaxation).

The tail gate is also of heavy sheet metal, its dimensions 2¾″ x 4″. A large slot is cut into this gate, from below upward, to allow protrusion of the tail and testicles. A small plate is used to close this slot in those instances, early in the training period, when a rat succeeds in turning around in the cage; without such a device, he is likely to gnaw at the edge of the tail gate.

For the most part, adult male rats have been used in this study, and most of the observations regarding heart rate levels and training regimes were made upon them. The training was facilitated by use of a battery of heart rate cages, since a preliminary rest of 15-20 minutes was found necessary before the heart rate reached basal levels. Generally, the records taken for experimental purposes, after training, were made on each rat separately, involving a 10-minute run following a 10-minute rest. Later, it was found that a restless animal could be prevented from communicating his mood to a neighbor by interposing a partition between adjacent cages. With this modification, a number of rats can be rested simultaneously and then each cage switched in sequence into the circuit for recording.

The heart rate adopted from any given run is the lowest one found in that tracing. The trend of succeeding determinations is followed from the curve of daily findings; that value indicated by a leveling off of the curve is taken as the "predictable,"

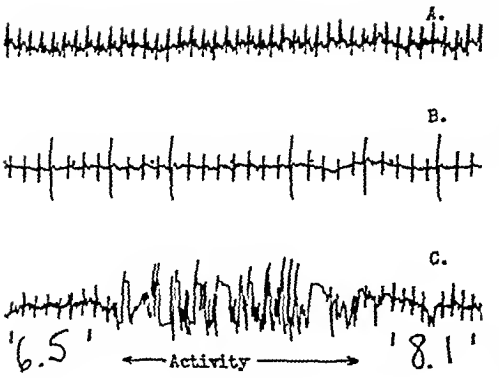

FIG. 3.

Illustrative tracings. Approximately one-half actual size.

a. Fairly complete ECG.

b. Ventricular extrasystoles (large, premature QRS complexes).

c. Effect of activity on heart rate (early phase of training).

if not always true basal, heart rate. The resultant values on 113 adult males, ranging from 200 to 370 g body weight, averaged 4.4±0.30 beats per second, or 264±18 per minute.

The attainment of a predictable heart rate level usually was found to require at least a month of training.

The course of training is fairly consistent in practically all instances, as indicated by 3 curves selected to bring out the average and the 2 extremes (Fig. 2). In general, the rats become progressively more and more

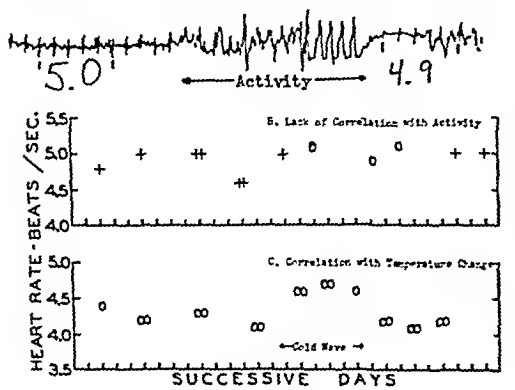

FIG. 4.

Factors altering heart rate.

a. Lack of effect of activity (late phase of training period).

b. Lack of day-to-day correlation with activity.

c. Effect of room temperature.

accustomed to the regime, and, though only a rare individual goes to sleep in the cage, most come to accept the restriction with equanimity. The symbols used on the curves are a purely subjective evaluation of activity as determined by inspection of the tracings.

The tracings obtained do not usually constitute complete electrocardiograms. A fair proportion of them do show the customary 3 major deviations of the cardiac cycle (Fig. 3a), but most record only a triphasic QRS complex. No doubt, establishment of better contact between the feet and the electrodes would suffice in all instances to bring out the P and T waves, if desired.

Occasionally, an individual rat exhibits cardiac arrhythmia, varying from a slight irregularity to a series of extra-systoles of apparently ventricular origin (Fig. 3b). In none of these animals was there noted any other sign of impaired health.

Any bodily activity is marked by large, irregular oscillations of the crystograph pen and so is easily distinguished on the tracing. In general, the heart rate varies with the degree and violence of movement (Fig. 3c).

However, it soon became clear that physical activity is not the only factor causing variations in heart rate, nor is it the most important factor. In a large proportion of subjects, neither the "basal" rate of an individual tracing nor the rates obtained on different days jibe as completely as one might predict with the state of rest or activity (Fig. 4b). Since a spontaneous quickening of the pulse often precedes a movement and since, in other instances, activity is neither accompanied nor followed by cardio-acceleration (Fig. 4a), it seems that greater variance is induced by the state of the emotions than by that of the musculature. The relative importance of this factor is further stressed by the uniform finding that increases in heart rate with body movement are of great degree only during the early phases of the training period.

Temperature changes likewise exercise an important influence on the heart rate. Much of this study was carried out in a constant temperature room at the University of Chi-

cago, and occasionally in wintertime, during a severe cold wave, the room temperature declined by several degrees. All heart rates taken during such periods were greatly elevated, even in rats which were resting quietly (Fig. 4c). In the investigation for which these heart rate studies were made,[10] one highly effective method used to accelerate the heart was chilling of the animal by depilation.

From these evidences it seems clear that dependable normal pulse rate determinations can be made in the unanesthetized rat, providing the extreme excitability of the animal and the great variability with temperature changes are recognized and steps taken accordingly. Early indications of studies in progress are that the training period may be cut to a week or two by the use of roomier cages than were formerly deemed necessary.

Summary. 1. A method is presented for accurate determination of the normal, conscious, resting heart rate in the albino rat. 2. The average value obtained on 113 adult males of 200-370 g body weight was 4.4± 0.30 beats per second, or 264±18 per minute. 3. Emotional states were found to cause greater variation in heart rate than muscular activity. 4. Alterations in environmental temperature proved to exert a decided influence on the rat heart rate.

[10] Moses, L. E., *Am. J. Physiol.*, 1944, **142**, 686.

15493 P

Effect of Alloxan upon External Secretion of the Pancreas.

M. I. GROSSMAN* AND A. C. IVY.*

From the Department of Physiology, Northwestern University Medical School, Chicago.

The report of Goldner and Gomori[1] that in dogs made "diabetic" by alloxan injection the intralobular duct cells of the pancreas showed vacuolization suggested to us the possibility that this might serve as a means of investigating whether these duct cells perform a secretory function.

Methods and Results. A standard preparation of secretin concentrate designated S_i[2] was used in these experiments. It is known to contain both secretin and pancreozymin. The particular lot of material used for these experiments was tested on 2 normal dogs and the threshold dose[3] was found to be 0.3 and 0.5 mg respectively, indicating that the material had standard potency. In 4 dogs rendered "diabetic" for 18 to 30 days by the intravenous injection of 75 mg of alloxan per kg of body weight, the threshold dose of this same lot of secretin concentrate was found to be 2, 4, 5 and 10 mg. The "diabetic state" was attested by the strongly positive sugar reaction of the urine accompanied by moderate decline in body weight.

Two dogs which were similarly treated with alloxan but failed to become "diabetic" showed a threshold of 0.4 and 0.8 mg secretin concentrate, respectively. In the 2 normal dogs mentioned above, which were used to establish the potency of the secretin preparation, the injection of alloxan in a dose of 75 mg per kg of body weight after the threshold response had been determined did not significantly alter their response to a subsequently administered threshold dose of secretin concentrate within 3 hours.

Amylase determinations by the method of Schmidt[4] were performed on all samples of

* Present address: University of Illinois College of Medicine, Chicago.

[1] Goldner, M. G., and Gomori, G., *Endocrin.*, 1943, **33**, 297.

[2] Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, 1938, **124**, 427.

[3] Ivy, A. C., Kloster, G., Drewyer, G. E., and Lueth, H. C., *Am. J. Physiol.*, 1930, **95**, 35.

[4] Schmidt, C. R., Greengard, H., and Ivy, A. C., *Am. J. Digest. Dis.*, 1934, **1**, 618.

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4 Schmidt, C. R., Greengard, H., and Ivy, A. C., *Am. J. Digest. Dis.*, 1934, **1**, 618.

TABLE I.
Secretin Threshold Dose and Amylase Concentration of Pancreatic Juice in Control Dogs and Dogs Treated with Alloxan.

Dog No.	Alloxan (mg/kg)	Days after alloxan	Urine sugar	Amylase (mg glucose/cc)	Threshold dose of secretin (mg)
1	none	—	0	1460	0.3
2	"	—	0	1280	0.5
3	75	18	++	1760	2.0
4	75	24	++	1120	10.0
5	75	28	+++	1690	5.0
6	75	30	++	1430	4.0
7	75	22	0	1260	0.4
8	75	30	0	1740	0.8

pancreatic juice but revealed no remarkable differences between the juice obtained from alloxan-treated animals and that of normal dogs (Table I). In one animal with alloxan-induced diabetes in which the pancreas was examined histologically, the vacuolization of the intralobular ducts described by Goldner and Gomori[1] was found; the acinar cells were normal.

Discussion. The finding of decreased responsiveness to secretin accompanied by histological evidence of damage to the duct epithelium[1] suggests but does not prove that the small duct cells participate in the formation of pancreatic juice. It is generally taught that the entire pancreatic juice is produced by the acinar cells; no secretory function is ascribed to the duct cells. In the salivary glands the duct cells are believed to contribute most of the liquid portion of the saliva.[5]

Two separate hormones, namely secretin and pancreozymin, control the secretory activity of the pancreas. The type of activity engendered by each hormone (water and bicarbonate secretion in the case of secretin; enzyme secretion in the case of pancreozymin) can vary independently. These facts are compatible with the concept that each hormone acts predominantly upon a different cell type in the pancreas, the secretin upon the intralobular duct cells and the pancreozymin upon the acinar cells.

A similar situation obtains in the gastric glands where the parietal cells produce hydrochloric acid and most of the water of the gastric juice while the granulated body chief cells provide the pepsin. The analogy between the gastric and pancreatic glands can be extended to include a functional parallelism, *to-wit*, the relationship between the rate of secretion of the juice and the concentration of the acidic or basic constituent respectively. In the stomach as the rate of secretion increases the concentration of hydrochloric acid increases and approaches a limiting value, namely the concentration of acid in the pure parietal secretion.[6] Similarly, as the rate of pancreatic secretion increases the bicarbonate concentration increases and approaches a limiting value, which, as in the case of the acid of the stomach, is approximately equal to the osmolar concentration of blood plasma.[7]

This concept of participation in elaboration of secretion by the small duct epithelial cells is offered as an hypothesis; much more evidence is required to establish or disprove its validity.

(Scanlon, Catchpole and Gersh[8] have also found a decreased responsiveness to secretin in alloxan diabetic dogs).

[5] Babkin, B. P., *Secretory Mechanism of the Digestive Glands*, P. B. Hoeber, New York, 1944.

[6] Gray, J. S., *Gastroenterology*, 1945, 1, 390.

[7] Hart, W. M., and Thomas, J. E., *Gastroenterology*, 1945, 4, 409.

[8] Personal communication from J. H. Scanlon, H. R. Catchpole, and I. Gersh, Naval Medical Research Institute, Bethesda, Md.

15494

Exposure of Guinea Pigs to Intermittent High Oxygen Tension and Its Failure to Depress Erythropoiesis.*

WILLIAM S. DONNELL, ARTHUR V. JENSEN AND HOWARD L. ALT.

From the Institute of Neurology and Department of Medicine, Northwestern University Medical School, Chicago, Ill.

In recent years the theory has been advanced that the rate of erythropoiesis is regulated by the oxygen tension in the bone marrow. It is well known that exposure to low oxygen tension over a sufficient period of time will produce a marked degree of polycythemia. However, there appears to be little unanimity of opinion among investigators about the effects of high oxygen tensions in man and animals.

Barach and McAlpin[1] found no significant alterations in the erythrocyte counts of patients with polycythemia vera following continuous exposure to 50% oxygen for 15 to 17 days. Reinhard, Moore, Duback and Wade[2] found that subjecting patients with sickle cell anemia to a continuous supply of 70-100% oxygen for 8 to 20 days caused a significant decrease in reticulocytes and erythrocytes. Campbell[3] exposed mice, rats, guinea pigs, rabbits, monkeys, and cats continuously to 40-60% oxygen for 18 to 57 days at normal barometric pressure. In all but the cats he observed a decrease in the number of erythrocytes. In 2 guinea pigs exposed for 57 days there was an average decrease of 35% in the number of erythrocytes and 22% in the amount of hemoglobin. The reports of other investigators who have worked on this problem (Karsner;[4] Archard, Binet and Le Blanc;[5] Binet, Bochet and Bryskier;[6] and Davis[7]) are unconvincing.

The purpose of the present investigation was to determine whether prolonged *intermittent* exposure of guinea pigs to high oxygen tension would cause a depression of erythropoiesis. Two of us[8] have previously shown that intermittent exposure to low oxygen tension produces a marked stimulation of erythropoiesis.

Materials and Methods. Fourteen young adult male guinea pigs weighing 300 to 400 g were used in the first experiment. Four animals served as controls and the remaining 10 were exposed to 80-100% oxygen at normal pressure 6 hours a day 6 days a week for 8 weeks. For the administration of high concentrations of oxygen, a gas-tight cylindrical tank of approximately 16 cu. ft. capacity was used. The tank was just large enough to contain a cage equipped with food and water for the experimental animals. It had 2 glass windows through which the animals could be observed during the exposure, and was equipped with trays of soda lime to absorb carbon dioxide. A constant internal temperature was maintained by the circulation of cool water through a system of coils. Commercially pure oxygen (U.S.P.) was supplied by continuous flow to the tank, and the flow was regulated by a pressure reduction valve. To avoid building up a positive pressure a needle valve connected directly to the wall of the tank, but at the opposite end from the oxygen inflow valve, was used as a constant bleeder. Careful adjustment of this valve served to balance the amount of outgoing air with the incoming oxygen. During

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Barach, A. L., and McAlpin, K. R., *Am. J. Med. Sc.*, 1933, **185**, 178.

2 Reinhard, E. H., Moore, C. V., Duback, R., and Wade, L. J., *J. Clin. Invest.*, 1944, **23**, 682.

3 Campbell, J. A., *J. Physiol.*, 1927, **63**, 325.

4 Karsner, H. T., *J. Exp. Med.*, 1916, **23**, 149.

5 Achard, G., Binet, L., and LeBlanc, A., *C. R. Acad. d. Sc.*, 1927, **184**, 771.

6 Binet, L., Bochet, M., and Bryskier, A., *J. de Physiol. et de Path. Gen.*, 1939, **37**, 524.

7 Davis, J. M., *J. Pharm. and Exp. Therap.*, 1943, **79**, 37.

8 Jensen, A. V., and Alt, H. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 384.

exposure, oxygen flowed into the tank at a rate of approximately 4.4 liters per minute.

After the experimental animals were sealed in the tank at the beginning of a daily exposure, the tank was flushed out with pure oxygen for 10 to 15 minutes, care being taken not to raise the tank pressure more than 10 mm Hg pressure over normal. The process of flushing required about 900 liters of oxygen. After flushing the oxygen concentration was determined. This initial concentration varied between 80 and 95% oxygen for all exposures. At the end of the 6-hour exposure period another oxygen concentration determination was made. This terminal concentration varied from 87 to 100% for all exposures. The average concentration of oxygen to which the animals were exposed was therefore about 92% which provided a partial pressure of oxygen of 699 mm Hg (4.4 times the normal). All oxygen concentration determinations were made by means of the Analox instrument.† Periodic observations of the experimental animals were made during exposures and at interval periods in an attempt to detect any abnormalities of behavior. The animals were weighed twice a week.

Blood studies were made on the experimental animals at the end of 183 hours exposure (covering 36 days) and at 287 hours (covering 57 days). The controls were studied at the same intervals. The animals were anesthetized with nembutal, and blood was drawn from the femoral artery 16 to 18 hours after periods of exposure to high oxygen tension. Erythrocyte counts were made with pipettes and hemocytometer chambers certified by the U. S. Bureau of Standards. The amount of hemoglobin was determined by the Duffie method[9] with an instrument calibrated by the oxygen capacity technic.

The second experiment was similar to the first except that the increase in partial pressure of oxygen was obtained principally by raising the barometric pressure in the tank. Six guinea pigs were subjected to 60 to 70% oxygen at 2 atmospheres pressure, which provided a partial pressure of oxygen of 998 mm Hg (6.3 times the normal). This was accomplished by first reducing the atmospheric pressure in the exposure chamber slowly to 400 mm Hg pressure below normal by means of an evacuation pump, and then gradually building up the pressure in the chamber with pure oxygen to one atmosphere over normal, and maintaining it there in the same manner as in the first experiment. The animals were exposed 6 hours a day 6 days a week, and received 17 exposures over a period of 20 days with a total exposure time of 106 hours. The number of erythrocytes and amount of hemoglobin were then determined.

Results. In the first experiment there was no significant change in the erythrocyte count and hemoglobin concentration after exposure to 80-100% oxygen at 1 atmosphere pressure. At the end of 183 hours of intermittent exposure covering a period of 36 days, the experimental animals showed a mean erythrocyte count of 5.29 (±.625) million per cu mm and a mean hemoglobin value of 12.9 (±.908) g per 100 cc. The control animals had a mean erythrocyte count of 5.11 (±.294) million per cu mm and a mean hemoglobin of 13.22 (±.511) g per 100 cc. The experiment continued with 9 experimental animals, one having died during the process of blood extraction presumably from an overdose of anesthetic. Blood was again taken from the animals at the end of 287 hours of intermittent exposure covering 57 days. The experimental animals then had a mean erythrocyte count of 5.22 (±.547) million per cu mm and a mean hemoglobin of 13.11 (±1.29) g per 100 cc. The controls had a mean erythrocyte count of 5.22 (±.646) million per cu mm and a mean hemoglobin value of 13.35 (±.983) g per 100 cc.

In the second experiment we also observed no change in erythropoiesis. After 106 hours of intermittent exposure to 50-60% oxygen tension at 2 atmospheres pressure the mean erythrocyte count for 6 animals was 4.81 (±.527) million per cu mm and the mean value for the amount of hemoglobin was 13.1 (±.538) g per 100 cc.

† Instrument produced by Oxygen Equipment and Service Co., Chicago, Ill.

[9] Duffie, D. H., *J. A. M. A.*, 1944, **126**, 95.

During both experiments the guinea pigs exhibited no adverse symptoms. They all ate readily, gained weight, and were normally active while in the tank and when they were put back into the storage cage with their controls during the interval period. No differences between the normal and the experimental animals could be observed.

Comment. Using the intermittent exposure method, it is apparent that high oxygen tension does not cause a depression of erythropoiesis in guinea pigs in contradistinction to the marked stimulation of erythropoiesis produced by low oxygen tension. With the continuous exposure method Campbell[3] observed significant reductions in the erythrocyte count and hemoglobin values in various species of animals. Because of the importance of this observation we exposed 6 guinea pigs continuously to 50-60% oxygen. Three animals died of pneumonia during the experiment. After 30 days, the remaining 3 animals had an average decrease of 21% in the erythrocytes and 13% in the amount of hemoglobin. Whereas these changes are not as great as those reported by Campbell, they further suggest that long continued exposure to oxygen may depress erythropoiesis in a normal animal.

The interesting experiment of Reinhard[2] *et al.* shows conclusively that erythropoiesis is depressed in patients with sickle cell anemia exposed to high oxygen tension. As such an effect was not obtained in patients with polycythemia vera (Barach and McAlpin)[1] it might be inferred that the hemopoietic equilibrium in sickle cell anemia is especially sensitive to increased oxygen pressures. The effect of prolonged high oxygen tension on the rate of erythropoiesis in animals and normal human beings warrants further investigation.

Summary. Guinea pigs were intermittently exposed to 80-100% oxygen at atmospheric pressure for 57 days, and to 60-70% oxygen at 2 atmospheres pressure for 20 days. The periods of exposure covered 6 hours a day 6 days a week. There was no significant diminution in the erythrocyte counts and hemoglobin values in any of these animals.

15495

The Effect of *Lithospermum* on the Mouse Estrous Cycle.*

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From the Haskins Laboratories, New York City.

Introduction. Based on a report[1] that certain American Indians have employed the ingestion of the herb *Lithospermum ruderale* as a conception preventative, Cranston[2] performed a series of experiments on mice and found that a fluid extract of *Lithospermum* when mixed with normal diet would rapidly induce a suppression of the estrous cycles as well as a lowered birth incidence in breeding females. From inferential evidence Cranston concluded that the active factor in the herb operates directly on the pituitary gland, suppressing the formation or release of gonadotropic hormone.

Since this type of "anti-hormonal" action

* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council. Thanks are hereby extended to Dr. H. B. Andervont of the National Cancer Institute for having supplied us with C_3H stock, and to Dr. S. H. Hutner of the Haskins Laboratories for many helpful suggestions relating to the problem under study.

1 Train, P., Hendricks, J. R., and Archer, W. A., *Medicinal Uses of Plants by Indian Tribes of Nevada*, Part II, p. 102. Issued by the Division of Plant Exploration and Introduction, Bureau of Plant Industry, U. S. Dept. of Agriculture, Washington, D.C., 1941.

2 Cranston, E. M., *J. Pharm. and Exp. Therap.*, 1945, **83**, 130.

is pharmacologically unique, work was undertaken (1) to confirm the effect of the drug on the estrous cycles of at least 2 unrelated strains of mice, and (2) to determine the effect of the drug on the incidence and development of mammary tumors, the appearance of which, in high tumor strains, is known to implicate the pituitary-ovarian endocrine system.

The latter study is still in progress. The former constitutes the subject of this note.

Experimental. Data were collected on 2 strains of mice: C_3H high mammary tumor females bred in this laboratory from stock obtained originally from the National Cancer Institute, and a heterozygous line of low-mammary-tumor albino mice.

Powdered *Lithospermum* from the whole plant, collected and dried in Montana, was fed in pellet form at a 15% level, the remaining 85% consisting of powdered Rockland Mouse Diet. The 15% level was chosen by extrapolation from a curve based on toxicity tests with groups of Rockland mice receiving 5%, 10%, 20%, 30% and 40% *Lithospermum* for a period of one month during which time a careful weight record was kept. The 15% level was the maximum dosage not affecting the weight of the fed animals.

Vaginal smears were made by the lavage method, with the use of distilled water and a fine pipette. The smears were dried under heat and stained with aqueous methylene blue. Differential counts were made under high dry magnification and approximately 5 fields were counted for each determination.

Twenty-four C_3H females 6-8 months of age were placed on the experimental diet and vaginal smears were taken daily for one month, after which smears were made weekly. Smears of 5 normal-diet females served as control. The animals were maintained on the diet for 3-5 months, during which time estrus occurred only rarely and in only a few of the mice. Generally speaking, the response in the C_3H strain to *Lithospermum* was immediate and persistent, in contrast to a type of refractoriness to the drug developed by some of the Rockland mice, described below.

Twenty Rockland females 2 months old were put on the 15% *Lithospermum* diet, with 10 females on normal diet serving as controls. Vaginal smears on the Rockland mice were taken daily for a period of 3 months. These smears were read differentially for percentage of cornified cells, nucleated epithelial cells and leucocytes. Four typical graphs of cornified cell levels are shown in Fig. 1. In contrast to the uniform response of the C_3H strain, the Rockland mice showed a great degree of individual variation, ranging from complete absence of response to full and continuous response. About 20% of the animals are in the former category, and 15% in the latter. The majority of the animals, however, (65%) went into an initial anestrus but came into estrus at about 30-day intervals with prolonged periods of anestrus between. Some of the mice at the end of several months developed a complete refractoriness to the drug, apparently recovering their normal cycles, though still on the *Lithospermum* diet, indicating either the development of an antagonist to the drug or an elevation of the organism's response threshold. Some evidence for the development of such a refractory state can be seen in 2 of Cranston's animals.[2]

Weights were taken on the Rockland animals at the conclusion of the experimental feeding. The control animals averaged 36.6 g and the *Lithospermum*-fed animals averaged 37.1 and 36.1 g, with no evidence of abnormal scatter. Cranston, using younger mice in experiments which involved administration of *Lithospermum* at rather higher dosage levels than employed by us, observed initial weight losses from which, however, the mice recovered while still on the diet.

The ovaries and uteri of females in *Lithospermum* anestrus showed marked atrophy, being about one-fourth normal size, with ovaries, especially in the C_3H strain, containing largely atretic follicles. Animals, however, which after long periods of anestrus became refractory to the drug and again developed estrus, had smaller than normal ovaries which, nevertheless, contained what appeared to be functional follicles.

Discussion. That a factor in *Lithospermum*

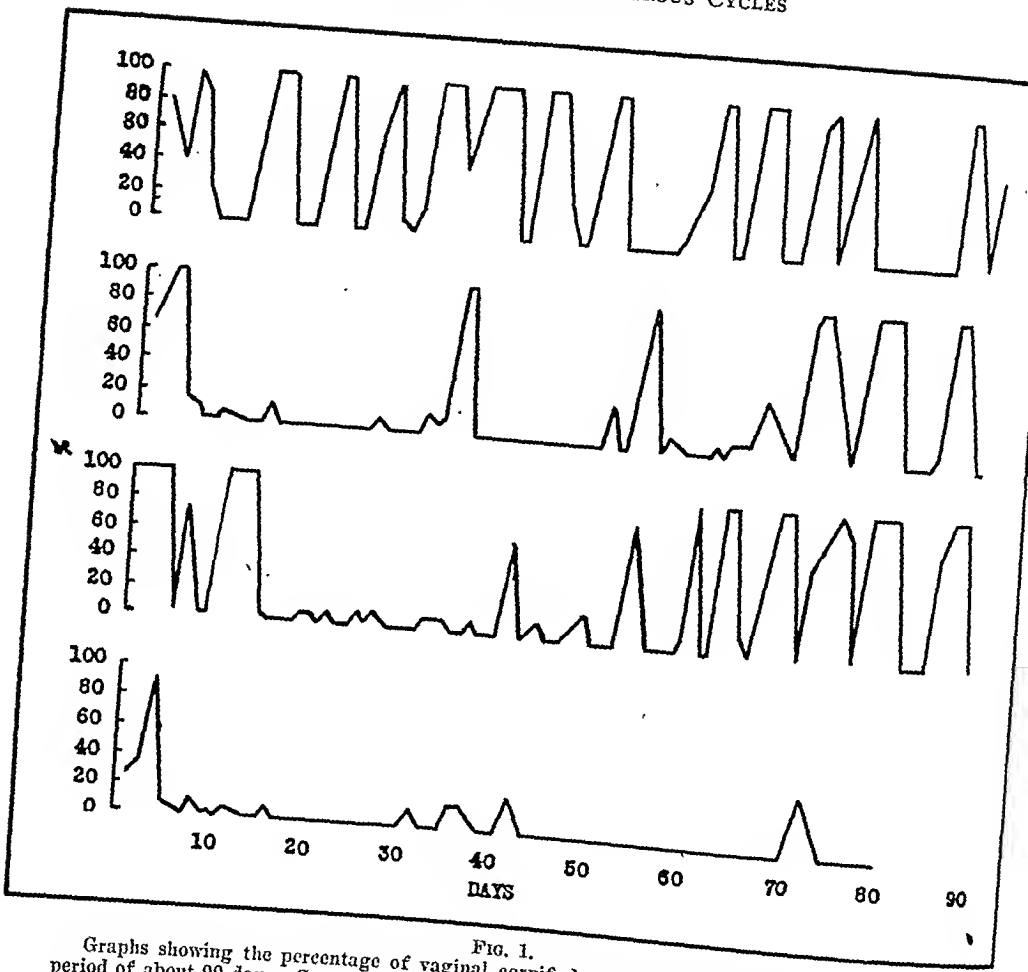

FIG. 1.

Graphs showing the percentage of vaginal cornified cells in female Rockland mice, over a period of about 90 days. Smears were made every 24 hours by the lavage method. The 4 sets of curves are: (top) of typical control mouse on standard diet; (two center) 2 examples of initial anestrus, followed by refractoriness in mice on a 15% *Lithospermum* diet; (bottom) example of persistent anestrus in mice on a 15% *Lithospermum* diet.

induces a suppression of estrous cycles in mice is confirmed. It is of interest to inquire into the nature of the mechanism of this action.

It is well known that a similar suppression of estrous cycles may result from a number of conditions, such as, inanition, deficiency of almost any of the vitamins, the action of injected "anti-hormone" substances, injected estrogens, androgens, progesterone, etc. It is doubtful, however, that *Lithospermum* operates through any of these media. General inanition may more or less be ruled out because of the fact that the *Lithospermum* effect can be achieved at dosages which do not affect body weight or general metabolic activity.[2] That a vitamin deficiency is involved is also doubtful. Vitamin A deficiency prevents the appearance of normal estrus and the condition is characterized by a persistent cornification of the vagina,[3] a symptom not present in animals in *Lithospermum* anestrus. Evans and Simpson[4] have produced permanent anestrus in rats by feed-

[3] Evans, H. M., and Bishop, K. S., *J. Metab. Research*, 1922, **1**, 335.

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ing a Vitamin B deficient diet. This anestrous condition yielded to implants of normal pituitary tissue, indicating that such a deficiency results in decreased gonadotropic potency of the pituitary. The exact mechanism by which vitamin E deficiency produces sterility is still subject to controversy; however, there is no doubt but that its effects are irreversible. The fact that a refractoriness to *Lithospermum* may develop in mice would seem to negate any hypothesis proposing that *Lithospermum* activity is dependent upon the induction of a deficiency of vitamins B and/or E.

The effect of estrogens and androgens in interfering with gonadotropic action cannot be considered to be similar to the *Lithospermum* effect because the latter is entirely free of estrogenic or androgenic potency.

That the mechanism of *Lithospermum* activity involves the thyroid has been doubted by Cranston[2] who found no significant change in the weights of the thyroid glands or in basal metabolic rate after *Lithospermum* treatment. This view has been confirmed by us through histological examination of the thyroids of normal Rockland mice and those of mice on a ration of 30% *Lithospermum* for a period of 5 months. In both cases the alveolar epithelium was low to cuboidal with a normal amount of colloid storage, the experimental animals showing a slightly greater amount of interstitial tissue. The question may be raised as to whether such a histological picture might be expected if *Lithospermum* acts in the manner of some of the goiterogens of plant origin, specifically the seeds of the *Brassica* genus. Griesbach[5] has shown that a brassica seed diet induces hyperplasia of the thyroid follicles accompanied by typical goiterogenic changes in pituitary histology, *e.g.*, increase in the number of basophils with hyalinization and vacuolization, and a decrease in the number of acidophils. After 56 days, however, this effect becomes less pronounced at which time colloid is again stored in the thyroid follicles. This might indicate the appearance of a refractoriness to the brassica seed diet similar to the refractoriness developed to *Lithospermum*. Since the histological material examined by us was taken at a time when refractoriness had undoubtedly developed, it is impossible to state with certainty that the thyroid is not involved. However, the bulk of the evidence would seem to point against its primary involvement.

Cranston[2] by indirect experimental procedures has quite convincingly ruled out specific action of *Lithospermum* on the other endocrine glands except the pituitary, concluding that the action is directly on this gland, which in her experiments underwent marked weight loss during *Lithospermum* feeding and the resulting anestrus.

The fact that some mice, especially of the Rockland strain, develop a refractoriness to the herb action suggests either that the active factor is antigenic and that the organism builds up a protective antibody mechanism, or that the pituitary (if it, in fact, be the tissue affected) builds up a rising threshold of reactivity. That the refractoriness, however, may come and go in an almost cyclic manner (Fig. 1) suggests the existence of an obscure mechanism which with the data at present available cannot be illuminated.

The assumption that the *Lithospermum* action is via the pituitary gland is the basis for the further study now in progress of the effect of the drug on mammary tumor development. It is established that in high mammary tumor strains ovarian hormone activity is essential to the development of tumors. The milk factor apparently cannot operate without an adequate hormonal substrate. In suppressing the ovarian endocrine tissues by negating the pituitary gonadotropins, one might expect to break a link in the chain leading to mammary tumor development. Experiments are now in progress to determine whether such a rationalization is valid.

Summary. Female mice of C_3H strain when fed on a diet of 15% *Lithospermum ruderale* go into an immediate and persistent anestrus. Female mice of Rockland strain fed similarly also go into anestrus which,

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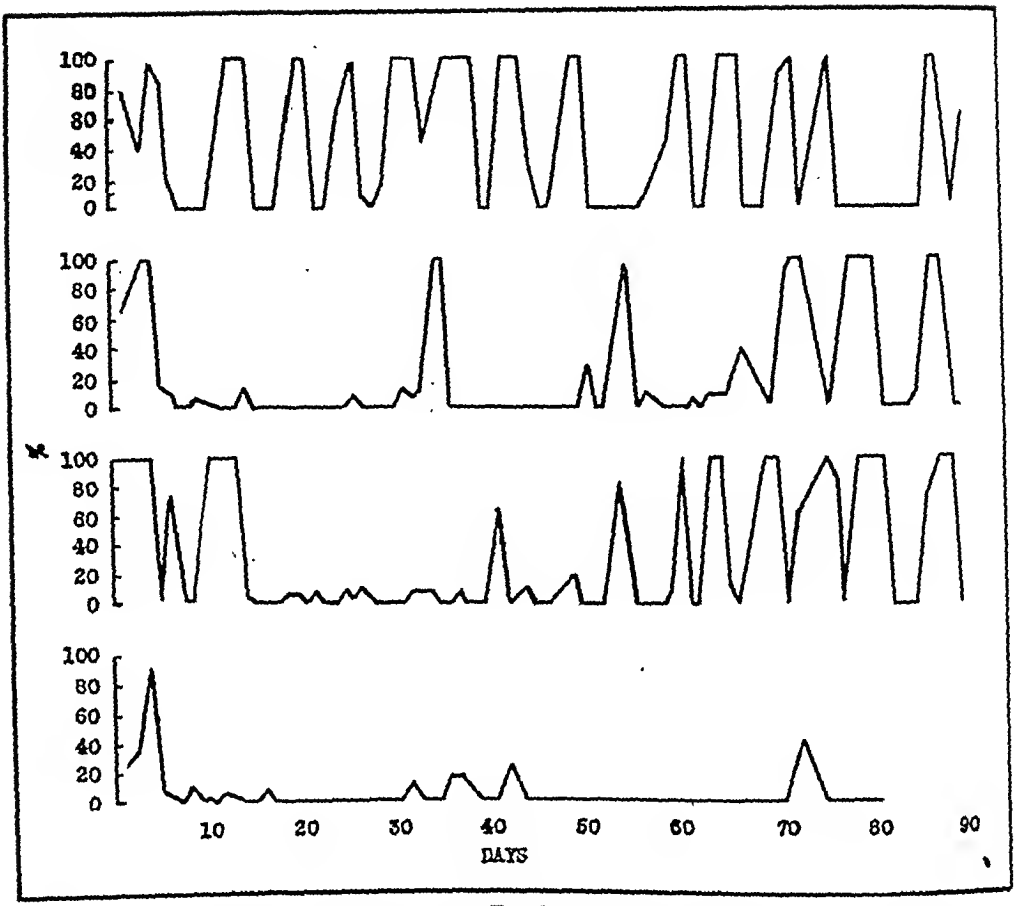

FIG. 1.

Graphs showing the percentage of vaginal cornified cells in female Rockland mice, over a period of about 90 days. Smears were made every 24 hours by the lavage method. The 4 sets of curves are: (top) of typical control mouse on standard diet; (two center) 2 examples of initial anestrus, followed by refractoriness in mice on a 15% *Lithospermum* diet; (bottom) example of persistent anestrus in mice on a 15% *Lithospermum* diet.

induces a suppression of estrous cycles in mice is confirmed. It is of interest to inquire into the nature of the mechanism of this action.

It is well known that a similar suppression of estrous cycles may result from a number of conditions, such as, inanition, deficiency of almost any of the vitamins, the action of injected "anti-hormone" substances, injected estrogens, androgens, progesterone, etc. It is doubtful, however, that *Lithospermum* operates through any of these media. General inanition may more or less be ruled out because of the fact that the *Lithospermum* effect can be achieved at dosages which do not affect body weight or general metabolic activity.[2] That a vitamin deficiency is involved is also doubtful. Vitamin A deficiency prevents the appearance of normal estrus and the condition is characterized by a persistent cornification of the vagina,[3] a symptom not present in animals in *Lithospermum* anestrus. Evans and Simpson[4] have produced permanent anestrus in rats by feed-

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however, may be followed by varying degrees of developed refractoriness to the drug, as evidenced in some mice by a reappearance of normal estrous cycles. The anestrous condition in both strains is accompanied by atrophy of the ovaries and uteri, and atresia of the follicles. Possible modes of action of the *Lithospermum* are discussed.

15496

Anesthesia. XXI. Anesthesia and the Steroid Hormones.*

DECAMP B. FARSON, C. JELLEFF CARR, AND JOHN C. KRANTZ, JR.
From the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md.

Selye[1-4] reported that the steroid hormones injected into certain species of laboratory animals produced anesthesia. In the generalization enunciated by Selye and referred to as "the fundamental law of steroid hormone anesthesia," this investigator states that "All compounds having a steroid-hormone action are capable of producing anesthesia while no compound devoid of hormone action possesses this power."[1] Indeed, Cashin and Moravek[5] had previously reported that they had anesthetized cats by injecting cholesterol suspensions intravenously. These experiments, in our opinion, appear to be inimical to the validity of Selye's generalization. In our studies on the effect of cholesterol on ether and pentothal sodium anesthesia, it appeared desirable to study further Selye's generalizations.

Experimental. Twenty-five rabbits received 2.5 cc/kg of 2% cholesterol suspension in a 25% solution of polyethylene glycol (Carbowax 1500) intravenously. Most of these animals appeared depressed. Six of them became unconscious and died promptly of acute pulmonary edema.

Fifteen dogs treated in a similar manner all manifested depression. Ten of these animals lost consciousness and died within 5 minutes.

Our attention was directed next to the hormonal steroids. Using the dosage schedules established by Selye we used the following compounds: progesterone, methyl testosterone, stigmasterol, estrone, benzestrol and diethylstilbestrol. Owing to the fact that in the "cat assay" of digitoxin, this glycoside in toxic quantities, produces a depression of the animal so that the anesthetic (ether) may be discontinued, we also used digitoxin in the series. In doses of 50 mg per rat weighing 92-205 g (average 158 g) not any of the foregoing compounds except progesterone and digitoxin produced "anesthesia."

Twenty-six animals, 8 male and 15 female, were used. None of the animals receiving digitoxin recovered from the comatose state produced by the drug. Three of the 9 animals receiving progesterone died.

Neither in the animals which died of progesterone nor with those that recovered was there ever a period in which the animal did not respond promptly to pain stimuli (pinching tail with hemostat). This syndrome cannot correctly be referred to as anesthesia when the sensory pathways are not blocked. In the rat, 6 mg/kg of

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pentobarbital sodium intraperitoneally as well as 3 to 4 cc of ether by inhalation abolish response to these stimuli.

Conclusion. We question the suitability of the word anesthesia either in its restricted or in its extensional meaning as applied to the syndrome of cholesterol depression. Etymologically "anesthesia" means without sensation, but usage confines it to a description of a reversible process; unless accidentally it becomes terminal. We hold, therefore, that when this syndrome of depression produced by these steroid compounds is described as anesthetic, that the use of the adjective lacks preciseness, and that this loose choice of the word is misleading.

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In a spontaneously ovulating form such as the rat the secretion of hypophyseal gonadotrophin (LH) may take place over a considerable portion of the estrous cycle before the concentration is great enough to produce ovulation. The present experiments were undertaken to determine whether, within a 2-hour period after the beginning of proestrus, there is a sufficient quantity of gonadotrophin in the blood to cause ovulation within the following 46 hours.

Materials and Methods. The regularity of the estrous cycles of a group of 70 young adult female rats of the Sprague-Dawley strain was determined by making daily vaginal smears during 2 or 3 cycles. On the day preceding the next calculated estrus the vaginal smears were made every 3 hours to determine more closely the onset of the proestrous phase. This procedure makes for a maximum error of 3 hours in determining the beginning of proestrus. As Long and Evans[1] have shown, the beginning of

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TABLE I.

Animal No.	Operation	No. tubal ova	No. corpora lutea	No. preovulatory follicles
1	None	4	4	1
5	"	6*	6	2
6	"	4	4	0
7	"	5	5	0
8	"	4	5	0
9	"	5*	5	0
2	Sham hypophyseet.	5	5	0
3	" "	6	7	0
4	" "	4	5	1
1	Hypophyseet.	0	0	4
2	"	0	0	5
3	"	0	0	5
4	"	0	0	2
5	"	0	0	6
6	"	0	0	5
7	"	0	0	3
8	"	0	0	4
9	"	0	0	4

* Number of ova difficult to determine because of beginning fragmentation.

proestrus is marked by the appearance of large numbers of uniform nucleated epithelial cells in the vaginal smear, to the exclusion of all other cell types. We adhered closely to the latter specification and did not regard as proestrous animals any individuals showing any degree of cornification of the vaginal epithelium, as determined by the vaginal smear method. Thus, from a group of 70 animals observed over a period involving 3 to 7 cycles, 18 animals were obtained which were within 3 hours of the onset of proestrus at the time of selection for experimental use.

Of this group of 18 early proestrous animals, 9 were hypophysectomized by the parapharyngeal approach within 2 hours of the time of selection. Of the 9 remaining animals, 6 were retained as unoperated controls and 3 were submitted to a sham hypophysectomy which included trephining of the sphenoid bone and tearing the dura mater. All animals were autopsied 48 hours following the onset of proestrus. The completeness of the hypophysectomy was checked by careful examination of the sella under the binocular microscope. The oviducts of all animals were ligated with fine silk at the tubo-uterine junction and the ovaries and tubes were then fixed in Bouin's solution.

Serial sections of one ovary and oviduct from each of the operated and control animals were stained with hematoxylin and eosin and prepared for microscopic study. The sections were examined carefully for the presence of incompletely formed corpora lutea, preovulatory type of follicles and for tubal ova.

Results and Discussion. The data are summarized in Table I. The ovaries of the animals which had been hypophysectomized in early proestrus all failed to ovulate, but from 4 to 7 follicles in the preovulatory phase were present.

In sham-operated and unoperated control animals microscopic study revealed from 4 to 7 tubal ova, a comparable number of incompletely formed corpora lutea and only an occasional preovulatory follicle. In some cases it was difficult to determine the exact number of tubal ova since their beginning fragmentation made it difficult to make an accurate count. Thus, in these cases, there was a slight discrepancy between the number of tubal ova and the number of incompletely formed corpora lutea.

The size of the residual cavity in the incompletely developed corpora of the sham-operated and control animals varied considerably. In some animals there was only a narrow rim of lutein tissue formed about a large follicular antrum, and in others there

was almost complete obliteration of the follicular cavity by newly formed lutein tissue. This variation in the development of the corpora lutea at a fixed time following the onset of proestrus is in keeping with the variation in the time of ovulation of different follicles within a single ovary.

The data support the conclusion that a hypophyseal hormone is required for ovulation and luteinization in the rat. It seems clear that this hormone must be secreted in sufficient quantities subsequent to the onset of proestrus, to produce ovulation and luteinization in the intact rat, as hypophysectomy in the early proestrus effectively prevents ovulation.

The procedure and results described in this paper provide the basis for further studies of the factors concerned with ovulation and corpus luteum formation in the rat.

Summary. Hypophysectomy of adult female rats within 2 hours after the beginning of proestrus prevents ovulation from occurring within a 46-hour postoperative interval. Sham hypophysectomy does not prevent ovulation as shown by the presence of partially developed corpora lutea and tubal ova. It is concluded that a hypophyseal hormone (LH) is required for ovulation and luteinization in the rat and that the quantity of hormone needed to produce ovulation and luteinization is secreted subsequent to early proestrus.

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Further Studies on Galactose Paralysis in the Rat.

B. H. ERSHOFF.

From the Emory W. Thurston Laboratories, Los Angeles, California.

The occurrence of paralysis has been observed in rats fed galactose on certain purified rations.[1] The purpose of the present experiment was to determine the effects of B vitamins on the incidence and severity of the above condition.

Procedure and Results. Female rats of the Sprague-Dawley strain were raised to maturity on a stock ration and selected for the present experiment at approximately 3 months of age and an average weight of 160 g (range 145 to 178 g). Four experimental rations were employed: diet I consisting of glucose alone; diet II of 50% glucose and 50% galactose; diet III of 50% glucose, 50% galactose and the following synthetic vitamins per kg of ration: thiamine hydrochloride 200 mg, riboflavin 400 mg, pyridoxine hydrochloride 20 mg and calcium pantothenate 200 mg; and diet IV of 40% glucose, 50% galactose and 10% yeast* providing the same amounts of the B vitamins as were present in diet III. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib.* feeding. Feeding was continued for 75 days or until death, whichever occurred sooner; and food and water intake determined daily for all rats.

Results are summarized in Table I. All animals fed galactose-containing diets developed a typical "galactose paralysis" consisting in early stages of an irregular and wobbly gait and progressing to the point of complete disuse of the hind limbs. One hundred per cent of the rats were affected, with severe paralysis developing in general by the 35th day. No significant differences in time of onset or severity of paralysis were observed on the various rations employed. Subsequent to the 35th day a rapid improvement occurred in the paralytic condition of rats fed the yeast-containing ration (diet IV); animals regained use of their limbs and by the 50th day no evidence of paralysis was observed in any of the surviving rats of this group. Recovery of paralyzed rats did not occur on the other rations

[1] Ershoff, B. H., *Am. J. Physiol.*, 1946, 147, 13.

* Hi-Ribo No. 24, Anheuser-Busch, Inc., St. Louis, Mo. Each gram contained the following vitamin potencies according to the manufacturer: thiamine, 2 mg; riboflavin, 4 mg; pyridoxine, 100-170 μg; pantothenic acid, 200-250 μg; and nicotinic acid, 400-500 μg.

TABLE I.
Summary of Experimental Data.

Group	No. of animals	Initial body wt (g)	Dead wt of decedents (g)	Survival (days)*	No. of rats paralyzed (%)	No. of rats recovered from paralysis (%)	Avg daily intake per rat during 1st mo. of exp. food (g)	Avg daily intake per rat during 1st mo. of exp. water (cc)
Diet I	8	159.2	76.4 (8)	41.2 ± 3.6	0	—	7.7	6.0
II	12	160.4	71.6 (12)	37.9 ± 4.7	100	0	12.8	38.5
III	6	160.8	72.2 (6)	47.3 ± 3.7	100	0	13.4	43.5
IV	8	159.7	88.2 (3)	67.8+†	100	87.5	15.8	59.7

The values in parentheses indicate the number of animals which died of which this is an average.

* Including standard error of the mean calculated as follows: $\sqrt{\epsilon d^2/n}$ / $\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

† The experiment was terminated on the 75th day of feeding at which time 5 of the 8 rats in this group were still alive.

employed. All animals fed galactose-containing rations developed mature bilateral cataracts from the 18th to 27th day of feeding, no significant differences being noted in time of appearance or the subsequent condition of the cataracts on the various diets employed. Paralysis and cataract were not observed in animals fed glucose alone (diet I). Food and water intake as well as length of survival was significantly greater on diet IV than on other rations tested.

Although yeast did not prevent the occurrence of paralysis, rats on diets containing this nutrient recovered rapidly from their paralytic condition in contrast to the continued paralysis in animals similarly treated but receiving their B vitamins in synthetic form. These results indicate (1) that the paralysis was reversible and (2) that some factor(s) in yeast other than thiamine, riboflavin, pyridoxine or pantothenic acid was responsible for the above effect.

Although paralysis may readily be demonstrated in rats fed galactose on certain purified rations,[1] this condition has not been reported for animals fed galactose on a more complete ration,[2,3] indicating that the purified diets employed were deficient in some factor necessary for the prevention of the paralytic condition. Attempts to prevent paralysis, however, with a single food supplement have been unsuccessful. Neither glucose, butter fat nor corn oil when fed in conjunction with galactose has prevented paralysis.[1] Unpublished work from this laboratory indicates that a diet of 50% casein† and 50% galactose was similarly ineffective. In addition neither thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, salt mixture‡ nor yeast* when fed with glucose and galactose was effective in this regard. Paralysis was readily prevented, however, by a diet of galactose 50.5%, casein† 25.0%, yeast* 10.0%, corn oil 10.0% and salt mixture‡ 4.5% together with vitamins A, D and E. Male and female rats of the Sprague-Dawley strain have been maintained in our laboratory for 9 weeks on the above diet with no adverse effects except the appearance of cataract. Similar results have been reported by Boutwell *et al.*[2] for a 6-week period in rats fed synthetic diets also containing galactose as the sole carbohydrate. Inasmuch as paralyzed rats in the

2 Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *Arch. Biochem.*, 1945, **7**, 143.

3 Mitchell, H. S., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 971.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Salt Mixture No. 1, Sure, B., *J. Nutrition*, 1941, **22**, 499. Paralysis was not prevented by diets containing 45% glucose, 50% galactose, and 5% of salt mixture.

present experiment recovered on diet IV in the absence of fat soluble vitamins, it would appear that these substances were not responsible for the protective effect of the above diets. The data suggests, therefore, either that "galactose paralysis" is due to a multiple deficiency developing when the various supplements were fed singly or that rations indicated above permitted the synthesis either by the animal's own tissues or its intestinal flora of substances capable of preventing paralysis.

Summary. Rats fed a diet consisting of glucose and galactose developed a severe flaccid paralysis of the hind limbs. Addition of yeast or synthetic B vitamins to the above diet failed to prevent the appearance of paralysis, although recovery subsequently occurred in animals receiving yeast.

15499

An Improved Rabbit Holder.

ROBERT E. SHIPLEY.

From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis, Ind.

In studies requiring repeated injections into the ear veins of rabbits, accidental extravasation with subsequent loss of the vein occurs not infrequently even under the best conditions. With the use of the conventional box-like rabbit holder there is rarely sufficient immobilization of the rabbit's head and ears to assure careful entry of the needle into the vein or to prevent its dislodgement by a sudden jerk of the animal's head.

Preliminary trials with a plaster mask shaped to accommodate the rabbit's head

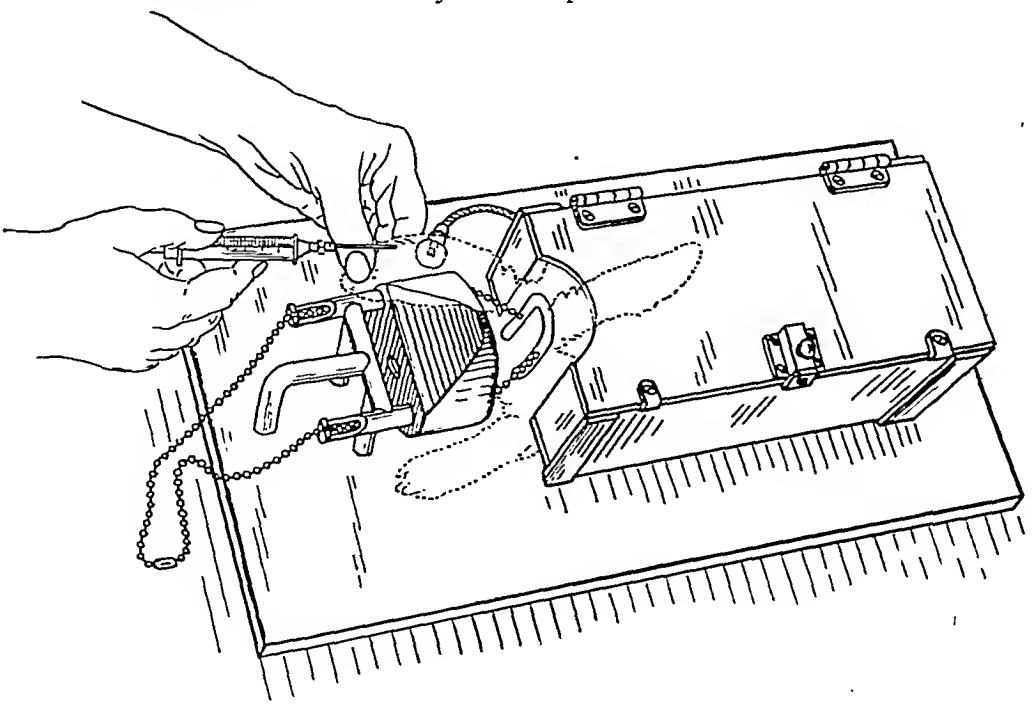

FIG. 1.

demonstrated that when the latter was rigidly supported the ears could be gently held and injected without mishap. After progressing through several models, a rabbit holder was designed according to the arrangement shown in Fig. 1, which aimed primarily at immobilization of the rabbit's head.

The head holder is made by molding a liquid thermo setting plastic* around a plaster of Paris model which has been shaped to conform to that of the head of a rabbit weighing about 2.5 kg. Provisions are made for leaving a hole from the nose to the outside. Within the 2 horizontal, tubular supports which are included in the plastic casting run two 3/16" ball chains 12 inches long. The anterior three-fourths of the rabbit's head is held in the cast by a horse-collar-shaped neckpiece to which are attached the ball chains. Once the rabbit's head is placed in the cast, the operator exerts traction on the chains, and under moderate tension the latter are secured by fitting them into 2 narrow vertical slots on the ends of the horizontal support tubes.

The body of the rabbit is confined in a 5" x 5" x 12" metal box with a hinged lid and clasp. Two flanges fastened to the front of the box prevent the rabbit's legs from reaching the area about the ears. All parts are mounted on a piece of 12" x 27" x ¾" plywood, the top and edges of which are covered with sheet metal 0.050" thick. For maximum rigidity, durability, and ease in cleaning, stainless steel, chrome-plated brass, and plastic were used. Wood and plaster of Paris are less durable but are otherwise adequate.

It has been found that by transilluminating the rabbit's ear from below, locating and visualizing particularly the smaller veins is greatly facilitated. With this procedure it is rarely necessary to clip or shave the hair over the vein in preparation for injection. A flexible arm with a small socket and bulb attached may be brought into any desired position beneath either ear.

The apparatus has been used several thousand times and has proved very satisfactory for rabbits weighing from 1.5 to 4 kg. Confining the rabbit in the holder causes no apparent discomfort.

Summary. A device is described which has been found to be of great advantage in effectively holding rabbits and immobilizing their ears for bleeding or injection procedures. The significant feature of the apparatus is a mask, shaped to accommodate the anterior three-fourths of the rabbit's head. An inverted, U-shaped collar holds the head firmly in the mask so that the position of the ears is correspondingly stabilized.

* "Catavar 101" (cream colored) or "Catavar 1001" (clear), Catalin Corporation of America, New York, N.Y. Plaster of Paris may also be used.

Acknowledgment is made to Mr. Clifford Wilson and to Mr. Roland Parker for suggestions in design.

15500

Retromammillary Inhibition of Cortically Induced Movement.

R. RHINES AND H. W. MAGOUN.

*From the Department of Anatomy, Northwestern University Medical School.**

Recent investigation has revealed 3 regions of the brain whose stimulation inhibits the response evoked from the motor cortex: cortical area 4-S,[1] the caudate nucleus,[2] and the bulbar reticular formation.[3]

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Dusser de Barrenne, J. G., and McCulloch, W. S., *J. Neurophysiol.*, 1941, **4**, 311.

2 Mettler, F. A., Ades, H. W., Lipman, E., and Culler, E. A., *Arch. Neurol. Psychiat.*, 1939, **41**, 984.

3 Magoun, H. W., and Rhines, R., *J. Neurophysiol.*, 1946, **9**, 165.

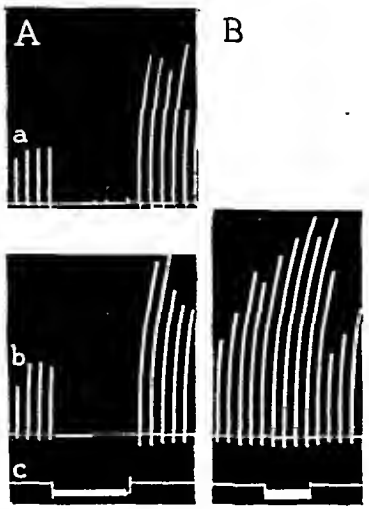

FIG. 1.
A. Cortically induced response of foreleg (a) and hindleg (b) inhibited during stimulation (c) of midbrain tegmentum. B. Patellar reflex (b) slightly augmented during stimulation (c) of same tegmental point.

During an experimental study of brain stem facilitation of cortically-induced movement,[4] it was observed that stimulation of a limited area of the rostral midbrain resulted in complete inhibition of the cortical motor response, while activation of a somewhat more extensive adjacent region produced depression of that response.

Methods. The left motor cortex of cats under light chlorolosane anesthesia was stimulated every 2 seconds by induction shocks and the responses of the right fore and hind legs were recorded with a kymograph. Using the Horsley-Clarke technic, the brain stem was stimulated in an exploratory fashion with 60 cycle sine wave current at intensities which did not by themselves evoke movement.

Stimulation of some of the points yielding inhibition was tested against the patellar reflex, elicited mechanically at 2-second intervals, and in one animal the midbrain tegmentum was stimulated against the flexor reflex of the foreleg, evoked every 2 seconds by exciting a cutaneous nerve.

[4] Rhines, R., and Magoun, H. W., *J. Neurophysiol.*, 1946, 9, 219.

Results. The predominant alteration of cortically-induced movement during stimulation of the upper brain stem consisted of facilitation.[4] However, as shown in Fig. 1A, the response to cortical stimulation ceased or was diminished when the sites indicated in Fig. 2 were stimulated. Depression of response (small circles) was obtained from the hypothalamus and overlying thalamus, and complete inhibition (large circles) was elicited from the midline region of the posterior hypothalamus, dorsal to the mammillary bodies (Fig. 2A), from the retromammillary region (Fig. 2A and B) and from the tegmentum of the rostral midbrain (Fig. 2A and C). Stimulation of this region also inhibited responses evoked from the bulbar pyramid.

Stimulation of the same sites that caused inhibition of the cortical motor response, produced no change, or in a few instances slight facilitation of the patellar reflex (Fig. 1B). The flexor reflex was, however, inhibited by stimulating the same area of the midbrain tegmentum which inhibited cortically-induced movement, and inhibition of the flexor reflex was also obtained by exciting the periaqueductal grey.

Though these same areas were explored in the brain stem of the monkey, inhibitory responses were not encountered.

Discussion. If the sites whose stimulation depressed or inhibited cortically-induced movement in these experiments belong to a single neural system, it would appear to be distributed diffusely in the posterior diencephalon, to be concentrated ventromedially in the most anterior part of the midbrain and to shift dorsolaterally into the tegmentum at more caudal midbrain levels. The presence of intermixed facilitatory elements may, however, have prevented detection of a possible further distribution. The subsequent augmentation of cortical motor response after inhibition, seen in Fig. 1A, might, for example, have been produced by combined stimulation of excitatory and inhibitory components.

A rostral midbrain inhibitory influence is of interest from the point of view of 3 release phenomena which have been found to fol-

demonstrated that when the latter was rigidly supported the ears could be gently held and injected without mishap. After progressing through several models, a rabbit holder was designed according to the arrangement shown in Fig. 1, which aimed primarily at immobilization of the rabbit's head.

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[3] Magoun, H. W., and Rhines, R., *J. Neurophysiol.*, 1946, **9**, 165.

15501

Effects of Ether and Curare on Neuromuscular Transmission.

WILLIAM SCHALLEK. (Introduced by Geo. H. Bishop.)

From the Laboratory of Neurophysiology, Washington University Medical School, St. Louis, Mo.

Anesthetists have noted that the amount of curare necessary to cause muscular relaxation is less in the presence of ether than of other anesthetics. Gross and Cullen[1] found that the contraction of the gastrocnemius muscle in response to electrical stimulation of its nerve is less in dogs anesthetized with ether than with other agents. They concluded that ether exerts a curariform action on neuromuscular transmission.

However these authors first determined the minimal strength of stimulus to which the muscle would respond, and then showed that after ether the muscle no longer responded to that strength of stimulus. Under these circumstances a failure of muscular contraction might be caused by a rise in the nerve threshold rather than by block of the neuromuscular junction. The present study avoids this difficulty by using maximal stimuli throughout. Each determination records the maximal response obtainable at that time.

In addition to the mechanical contraction, the electrical response of the muscle was also measured. This permits differentiation of the effect of ether on the contractile mechanism from its effect on the muscle action potential.

Method. The following studies were made on rats. The animals were anesthetized through a tracheal cannula attached to an ether bottle. The bottle was provided with a valve which controlled the amount of ether in the inspired air. Animals were kept lightly etherized throughout an experiment; when the effect of ether was to be studied the valve was turned to permit the maximal amount of ether to be drawn into the lungs. If natural respiration stopped, artificial respiration was applied by attaching an intermittent air pump to the side arm of the cannula. Natural breathing was usually resumed after a few seconds' artificial respiration.

In the experiments on curare Intocostrin (Squibb) was used. One cc of this preparation is described as containing the equivalent of 20 units of standard drug. It was injected into the femoral vein. The mechanical response was recorded through a lever attached to the tendon of the gastrocnemius, while the electrical responses were led off by needle electrodes inserted into the muscle. The height of potential was measured on an oscilloscope.

Results. The maximal etherization possible with the apparatus used blocks respiration before neuromuscular transmission. After 12 minutes respiration stops, while the electrical response of the muscle to maximal stimulation of its nerve shows an average depression of 12% (8 to 15) in 5 experiments. It is possible that the greater depression obtained by Gross and Cullen is due to their recording the mechanical contraction of the muscle. However a record of the mechanical response to maximal stimulation showed a depression of only 9% after 20 minutes of deep etherization. More probably the different results of Gross and Cullen are assignable to their use of minimal stimuli.

Since ether causes respiratory depression, the decreased muscular response might be due to anoxia rather than to direct action of ether. This possibility was tested by having the lightly etherized animal rebreathe into a balloon attached to the tracheal cannula. This kept the ether concentration reasonably constant, but permitted the oxygen concentration to drop rapidly. Two experiments showed an average depression of the muscle action current of 5% in 12 minutes. As a further check, after recovery the same animals were heavily etherized and oxygen was then blown in through the ether bottle. This increase in oxygen with little if any change in ether concentration caused an average recovery of 5% in 9 minutes. It appears

[1] Gross, E. G., and Cullen, S. C., *J. Pharm. and Exp. Therap.*, 1943, **78**, 358.

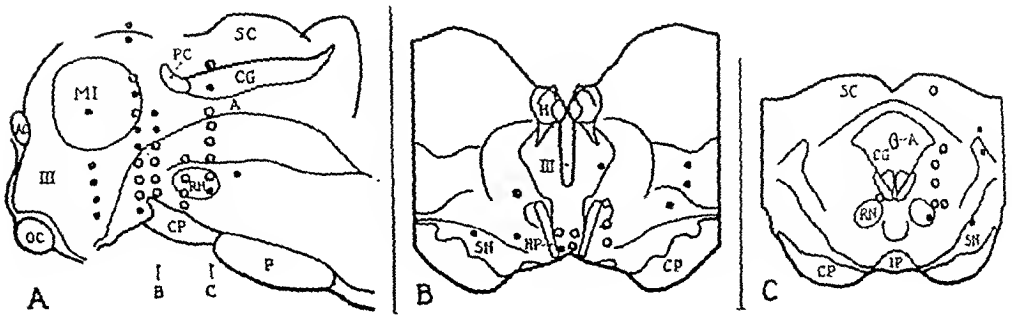

FIG. 2.

Distribution of sites whose stimulation completely inhibited (large circles) or depressed (small circles) cortically induced leg movement. A. Midsagittal reconstruction of cat's brain stem. B. Transverse section of retromammillary region. C. Transverse section of midbrain. Levels B and C are indicated by arrows in A.

A—Aqueduct
AC—Anterior commissure
CG—Central gray
CP—Cerebral peduncle
H—Habenula
HP—Habenulo-peduncular tract
IP—Interpeduncular nucleus
MI—Massa intermedia
OC—Optic chiasm
P—Pons
PC—Posterior Commissure
RN—Red nucleus
SN—Substantia nigra
III—Third ventricle

low injury to the mesencephalon. First, since Sherrington's discovery of decerebrate rigidity, it has been thought that one of the chief inhibitory centers from which lower brain stem levels are released in this condition is located in the midbrain. Ingram and his associates have made it clear that the red nucleus is not the exclusive midbrain inhibitory component eliminated by decerebration, as was once believed, though some rigidity does follow destruction of the red nuclei.[5] The sites yielding inhibition in the present study bear no obvious topographic relation to the red nucleus.

Second, a release phenomenon described as "obstinate progression" has been shown by Bailey and Davis[6] to follow destruction of the interpeduncular nucleus. Ventromedial midbrain sites whose stimulation yielded inhibition in the present experiments were located just rostral to the interpeduncular nucleus, but inhibitory responses were not obtained from this nucleus itself, nor from the habenulae or positions that were exclusively referable to the habenulo-peduncular tract leading from it to the interpeduncular nucleus.

Third, ventromedial retromammillary lesions have been shown by Ingram, Barris and Ranson[7] to produce catalepsy in the cat. A large number of the inhibitory responses elicited in the present experiments were obtained from the area destroyed in such cataleptic animals, but to what extent injury to these inhibitory elements was involved in producing the cataleptic syndrome is at present uncertain.

Summary. A rostral midbrain area, the stimulation of which inhibits cortically-induced movement in the cat, has been described.

[5] Ingram, W. R., and Ranson, S. W., *Arch. Neurol. Psychiat.*, 1932, **28**, 483.

[6] Bailey, P., and Davis, E. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 307.

[7] Ingram, W. R., Barris, R. W., and Ranson, S. W., *Arch. Neurol. Psychiat.*, 1936, **35**, 1175.

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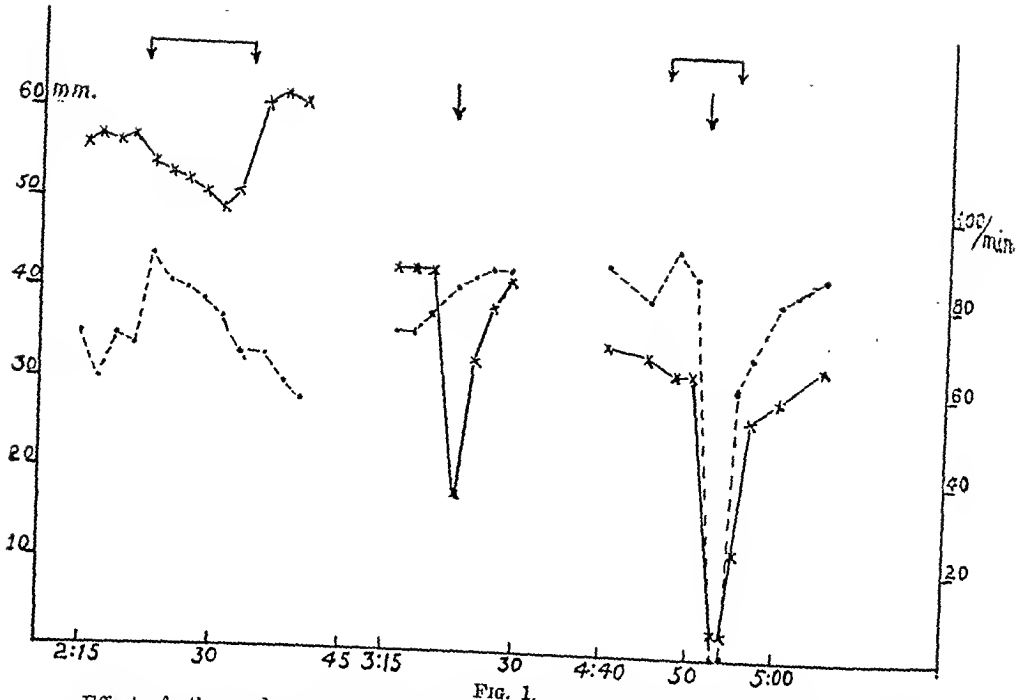


FIG. 1.

Effect of ether and curare on muscle action potential and respiratory rate in the rat. A progressive decline in the height of the action potential occurs during a long experiment, presumably due to deterioration of the muscle substance around the needle electrodes. Other experiments in which the order of the tests was varied show that this decline does not affect the conclusions. Intocostrin administered 0.1 unit per kg. Dash curves, rate of respiration, scale per minute at right margin. Full curves, height of muscle spike, scale at left margin in mm. Time on base line. Light ether throughout except between double arrows, deep ether. At single arrows, curare administered.

then that of the 12% depression following etherization, 5% may be caused by asphyxia.

The remaining depression is not enough to account for the observed increase of the action of curare in the presence of ether. That there is a synergism between the two agents is seen in Fig. 1. The maximal dose of ether alone caused a 14% depression of muscle action potential in this experiment, while 0.1 unit curare/kg caused an average depression of 46%, with minimal ether. Although the sum of the depressions caused by these agents given separately is 60%, the same dose of curare given 3 to 5 minutes after deep etherization has begun caused a depression of 91%. When the same dose of curare was given one minute after deep etherization began, the depression was only 72%, indicating that the ether had not yet reached the optimum concentration at the site of action.

In addition to this effect, a synergic action on respiration is shown by the same experiment. Ether alone stops respiration in 12 minutes, while this dose of curare used alone has no significant effect on respiration. But if this dose is given 3 to 5 minutes after deep etherization has begun, respiration stops 2 minutes after curare is injected.

Discussion. Although Gross and Cullen reported complete neuromuscular block under ether as tested by minimal stimuli, only a slight decrease of amplitude is found in the present study when maximal stimuli are used. This suggests that the depression which the above workers obtained may have been due to increase in the nerve threshold. The present paper does not permit localization of the peripheral action of ether. Since the depression obtained in the mechanical response was no greater than that obtained

in the electrical response, ether evidently acts proximal to the contractile process of the muscle. The synergism shown between ether and curare might suggest that they act at the same site, but this does not necessarily follow. Since we do not yet know the exact point at which curare acts, further speculation as to the peripheral action of ether does not seem warranted.

As noted above, curare has been reported to cause greater "relaxation" of muscles during anesthesia with ether than during the same degree of anesthesia with other agents. While this suggests that there is a greater synergism of curare with ether than with these other agents, another possibility exists. Anesthetists judge the degree of anesthesia to a large extent by the respiration, and amounts of ether and other agents which depress the respiratory center to the same degree may have different effects at other centers, as well as on the neuromuscular junction. A detailed examination of the actions of various anesthetics would be necessary to settle this point.

Summary. The response of the gastrocnemius to maximal stimulation through its nerve was studied in the rat. During deep etherization there is a depression of 12% in the muscle action potential. At the same time the respiration is depressed; anoxia alone due to this may account for 5% depression. Hence a muscular depression of only 7% can be attributed to the action of ether, not necessarily involving neuromuscular block. Nevertheless there is a synergism between ether and curare, since the effects of these agents given together is one-third greater than the sum of their separate effects.

Acknowledgment is due Dr. Geo. H. Bishop for advice in this research, and to Dr. S. M. Walker for assistanee. E. R. Squibb & Sons furnished the intocostrin used.

15502

Effect of Enzyme Inhibitors on Transformation of Enzymes in the Living Cell.

JOHN M. REINER. (Introduced by M. B. Visscher.)

From the Department of Physiology, The Medical School, University of Minnesota.

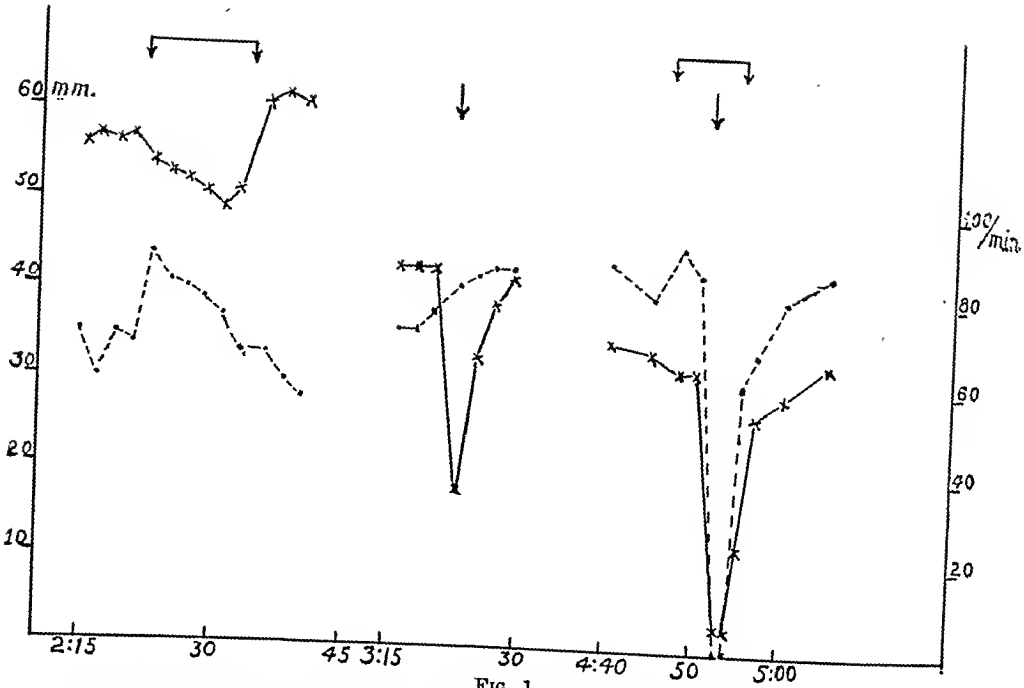
Transformations in the enzymatic constitution of the living cell may occur as a result of gene mutation or as a physiological transformation in the presence of a constant genome. Changes of the latter type are most easily detected and studied in the cells of microorganisms, which are less specialized and more flexible than the cells of mature multicellular organisms, and present fewer complicating factors than the closely interrelated complex of cells found in the embryo. These changes have long been familiar to microbiologists; but they have until recently been the subject of much controversy, centering around the question whether they are only apparent transformations resulting from the action of natural selection upon pre-existing mutants in microbiological populations. Recent developments in the genetics of yeast[1] have made it possible to settle the controversy at least for this group of organisms; and it has been demonstrated by Spiegelman and coworkers[2,3] that modifications of enzyme constitution take place in genetically stable yeast strains, without the intervention of natural selection and without cell multiplication.

Most domestic strains of yeast readily ferment glucose, but do not ferment galactose. Some of them can, however, acquire the ability to ferment galactose after

[1] Lindegren, C. C., *Bact. Rev.*, 1945, **9**, 111.

[2] Spiegelman, S., Lindegren, C. C., and Hedgecock, L., *Proc. Nat. Acad. Sci.*, 1944, **30**, 13.

[3] Spiegelman, S., and Lindegren, C. C., *Ann. Mo. Bot. Gard.*, 1944, **31**, 219.



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Discussion. Although Gross and Cullen reported complete neuromuscular block under ether as tested by minimal stimuli, only a slight decrease of amplitude is found in the present study when maximal stimuli are used. This suggests that the depression which the above workers obtained may have been due to increase in the nerve threshold. The present paper does not permit localization of the peripheral action of ether. Since the depression obtained in the mechanical response was no greater than that obtained

the electrical response, ether evidently ts proximal to the contractile process of e muscle. The synergism shown between her and curare might suggest that they act the same site, hut this does not necessarily llow. Since we do not yet know the exact oint at which curare acts, further specula- on as to the peripheral action of ether does ot seem warranted.

As noted ahove, curare has been reported cause greater "relaxation" of muscles dur- g anesthesia with ether than during the ame degree of anesthesia with other agents. hile this suggests that there is a greater nergism of curare with ether than with ese other agents, another possihility exists. nesthetists judge the degree of anesthesia a large extent by the respiration, and mounts of ether and other agents which epress the respiratory center to the same egree may have different effects at other enters, as well as on the neuromuscular junction. A detailed examination of the actions of various anesthetics would be necessary to settle this point.

Summary. The response of the gastrocnemius to maximal stimulation through its nerve was studied in the rat. During deep etherization there is a depression of 12% in the muscle action potential. At the same time the respiration is depressed; anoxia alone due to this may account for 5% depression. Hence a muscular depression of only 7% can be attributed to the action of ether, not necessarily involving neuromuscular hlock. Nevertheless there is a synergism between ether and curare, since the effects of these agents given together is one-third greater than the sum of their separate effects.

Aeknowledgment is due Dr. Geo. H. Bishop for advice in this research, and to Dr. S. M. Walker for assistance. E. R. Squibb & Sons furnished the intocostrin used.

15502

Effect of Enzyme Inhibitors on Transformation of Enzymes in the Living Cell.

JOHN M. REINER. (Introduced by M. B. Visscher.)

From the Department of Physiology, The Medical School, University of Minnesota.

Transformations in the enzymatic constitution of the living cell may occur as a result of gene mutation or as a physiological transformation in the presence of a constant genome. Changes of the latter type are most easily detected and studied in the cells of microorganisms, which are less specialized and more flexihle than the cells of mature multicellular organisms, and present fewer complicating factors than the closely interrelated complex of cells found in the embryo. These changes have long been familiar to microbiologists; but they have until recently been the subject of much controversy, centering around the question whether they are only apparent transformations resulting from the action of natural selection upon pre-existing mutants in microbiological populations. Recent developments in the genetics of yeast[1] have made it possible to settle the controversy at least for this group of organisms; and it has been demonstrated hy Spiegelman and coworkers[2,3] that modifications of enzyme constitution take place in genetically stable yeast strains, without the intervention of natural selection and without cell multiplication.

Most domestic strains of yeast readily ferment glucose, but do not ferment galactose. Some of them can, however, acquire the ahility to ferment galactose after

[1] Lindegren, C. C., *Bact. Rev.*, 1945, 9, 111.

[2] Spiegelman, S., Lindegren, C. C., and Hedgecock, L., *Proc. Nat. Acad. Sci.*, 1944, 30, 13.

[3] Spiegelman, S., and Lindegren, C. C., *Ann. Mo. Bot. Gard.*, 1944, 31, 219.

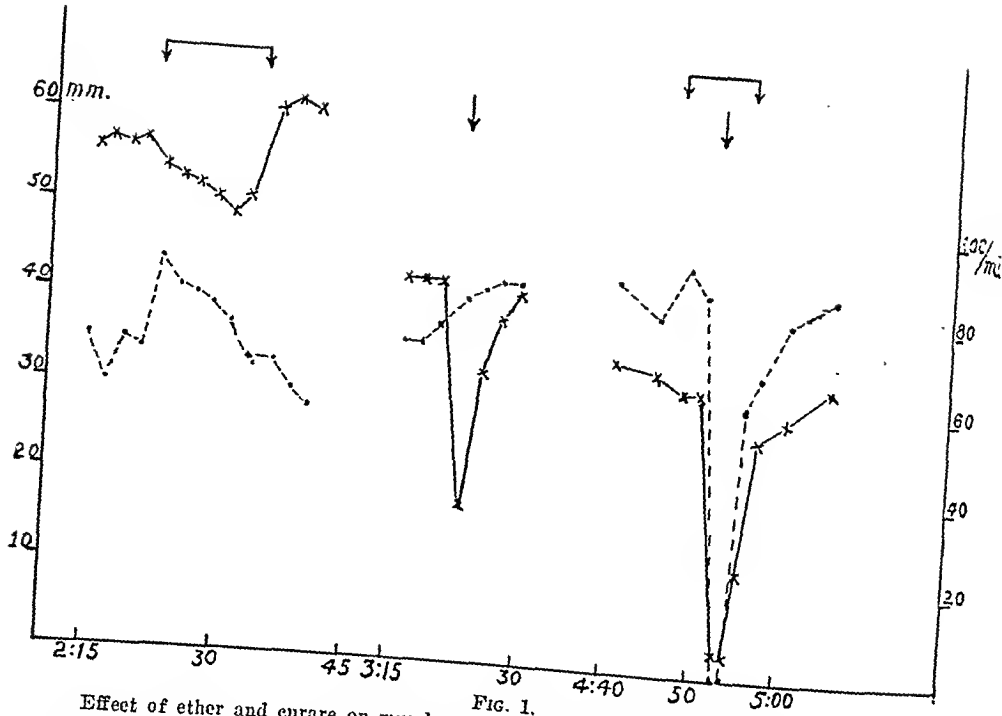

FIG. 1.

Effect of ether and curare on muscle action potential and respiratory rate in the rat. A progressive decline in the height of the action potential occurs during a long experiment, presumably due to deterioration of the muscle substance around the needle electrodes. Other experiments in which the order of the tests was varied show that this decline does not affect the conclusions. Intocostrin administered 0.1 unit per kg. Dash curves, rate of respiration, scale per minute at right margin. Full curves, height of muscle spike, scale at left margin in mm. Time on base line. Light ether throughout except between double arrows, deep ether. At single arrows, curare administered.

then that of the 12% depression following etherization, 5% may be caused by asphyxia.

The remaining depression is not enough to account for the observed increase of the action of curare in the presence of ether. That there is a synergism between the two agents is seen in Fig. 1. The maximal dose of ether alone caused a 14% depression of muscle action potential in this experiment, while 0.1 unit curare/kg caused an average depression of 46%, with minimal ether. Although the sum of the depressions caused by these agents given separately is 60%, the same dose of curare given 3 to 5 minutes after deep etherization has begun caused a depression of 91%. When the same dose of curare was given one minute after deep etherization began, the depression was only 72%, indicating that the ether had not yet reached the optimum concentration at the site of action.

In addition to this effect, a synergic action on respiration is shown by the same experiment. Ether alone stops respiration in 12 minutes, while this dose of curare used alone has no significant effect on respiration. But if this dose is given 3 to 5 minutes after deep etherization has begun, respiration stops 2 minutes after curare is injected.

Discussion. Although Gross and Cullen reported complete neuromuscular block under ether as tested by minimal stimuli, only a slight decrease of amplitude is found in the present study when maximal stimuli are used. This suggests that the depression which the above workers obtained may have been due to increase in the nerve threshold. The present paper does not permit localization of the peripheral action of ether. Since the depression obtained in the mechanical response was no greater than that obtained

being 90 minutes, data obtained from unadapted cells are given only from 90 minutes on. With fully adapted cells, only the highest inhibitor concentration was used. Table I shows a typical set of results.

Despite the fact that the O_2 consumption (not shown) was just as completely inhibited by 10^{-3} M azide in adapted as in unadapted cells, the fermentation of galactose by adapted cells was not only not inhibited, but was actually stimulated by azide. None of the other compounds used gave such results; and we therefore present the data for them somewhat more briefly. Concentration curves were obtained, but we show here only the data obtained at the highest concentrations used. The stimulatory effect of azide at low concentrations will be discussed more fully elsewhere.

As in the case of azide, the effect of these compounds on O_2 consumption was very nearly the same for adapted and unadapted cells. Unlike azide, however, they inhibited the galactose fermentation equally well in both cases, with the exception of DNP. The results therefore leave open the question of the mechanism of inhibition.

TABLE II.
Effect of NaF, IAA, and DNP on Galactose Fermentation.
(Excess CO_2, % of control).

NaF 2×10^{-2} M	IAA 2×10^{-4} M	DNP 10^{-3} M
	Unadapted cells.	
0	14	3
	Adapted cells.	
6	0	40

We therefore attempted to determine whether the inhibition of unadapted cells could be reversed. Cells were exposed to the inhibitors for 90 minutes in the presence of galactose, then carefully washed free of adhering solutions, and tested for fermentation on fresh galactose. If any adaptation had occurred, the washed cells should have begun to ferment before 90 minutes. The results from 60 minutes onward are given.

With the possible exception of fluoride, the results appear negative. In the case of IAA, however, irreversible changes tend to occur after long periods of exposure. Fluoride[9] is known to combine with magnesium and phosphate as a complex salt within the cell, this complex then uniting with the enzyme enolase; and it is not certain that this complex would have been removed by washing the cells. Consequently, the results of Table III must be regarded with some reservations.

TABLE III.
Reversibility of Inhibition of Adaptation.
(Excess CO_2, % of control).

NaF 2×10^{-2} M	IAA 2×10^{-4} M	DNP 10^{-3} M
17	2	3

Discussion. The most clearcut results with inhibitors have been obtained with sodium azide. This poison prevents adaptation from occurring, but does not prevent galactose fermentation after adaptation has been allowed to take place. It does not interfere with the metabolic chain by which galactose is transformed into alcohol and CO_2. It must therefore prevent the formation of one or more essential enzymatic or coenzymatic links in that chain. This supposition is reinforced by the knowledge that azide does in fact inhibit synthetic processes, and does not inhibit normal glucose fermentation.

The poison DNP inhibits adaptation completely, but inhibits galactose fermentation by adapted cells to the extent of only 60%. It is known to have an effect on synthesis[10] even at rather low concentrations; and it may in these experiments be functioning in a mixed way—to inhibit synthesis of the adaptive enzyme or enzymes, and to block or slow up part of the process of fermentation.

The compounds NaF and IAA are known to block the Meyerhof fermentation scheme.[9,11] It is therefore likely that they merely prevent the adaptive enzymes from manifesting themselves, without necessarily preventing the adaptive enzymes from being formed.

[9] Warburg, O., and Christian, W., *Bioch. Z.*, 1942, **310**, 384.

[10] Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1943, **21**, 77.

[11] Lundsgaard, E., *Bioch. Z.*, 1930, **217**, 162.

TABLE I.
Effect of NaN_3 on Galactose Fermentation.

Time	Azide conc.:	Control	10^{-5} M	10^{-4} M	10^{-3} M
		Unadapted cells.			
90-180′	Microlitres	138	242	28	26
	% of control	—	175	20	19
		Adapted cells.			
10-120′	Microlitres	944			1314
	% of control	—			139

a period of exposure to this sugar under aerobic conditions. The time required for "adaptation" (a term introduced by Karström[4] to denote this process) is a characteristic constant for each strain of yeast. It is this enzyme transformation which has been most extensively studied by Spiegelman, and which is the subject of the present report.

The effect of several well-known enzyme inhibitors on the adaptation to galactose fermentation has been studied in the hope of casting further light on the nature of the process. In particular, we wished to obtain evidence bearing on the following crucial question: Is adaptation the synthesis of one or several new enzymes or coenzymes by the cell, or is it merely a lag within the metabolic cycle of the substrate itself, involving the formation of a certain level of intermediate products (*e.g.*, phosphorylated intermediates), as in the well-known induction period which occurs when glucose is fermented by dried yeast? Our results point strongly to the former alternative—the occurrence of synthesis.

Materials and Methods. A pure strain of *Saccharomyces carlsbergensis* (CLD-1A, obtained from Dr. S. Spiegelman) was grown in liquid culture containing peptone and 8% glucose, together with yeast extract and the necessary salts. For experiments, 48-hour cultures were harvested, washed several times with M/15 KH_2PO_4 to remove adherent medium, and finally suspended in enough M/15 KH_2PO_4 to give about 20 mg (wet weight) of yeast in 1 ml of suspension. Aliquots of 1 ml were distributed among conventional Warburg reaction vessels; enough galactose solution to make a final concentration of 4% was placed in the side-arm of each vessel; and enough distilled water, inhibitor or phosphate solution was added to the main chamber to bring the final volume of liquid to 2 ml. In occasional experiments the concentration of yeast or of galactose was varied, but in any one experiment the conditions in all vessels were strictly comparable. After equilibration on the Warburg bath, the substrate was tipped into the main chamber, the time at which this occurred being designated as zero time. Oxygen consumption and CO_2 production were followed at 15-minute or sometimes at 10-minute intervals by the usual 2-cup method.[5] The excess of CO_2 production over O_2 consumption in a given time period was taken as an index of fermentation and hence of adaptation rate. Inhibitor solutions were adjusted to a pH of 4.5 before use.

Experimental Results. The inhibitors chiefly used were sodium azide (NaN_3), sodium fluoride (NaF), 2-4-dinitrophenol (abbreviated DNP), and iodoacetic acid (abbreviated IAA). Their effects on unadapted and fully adapted cells were examined. Adapted cells were obtained by growing the yeast in the usual liquid medium with galactose substituted for glucose.

Azide is known as an inhibitor of hemin catalyses,[6] but also (unlike HCN and H_2S) as a specific inhibitor of syntheses even under anaerobic conditions.[7,8] This makes it particularly useful for our purpose.

The adaptation time of our yeast strain

[4] Karström, H., *Erg. Enzymforsch*, 1938, **7**, 350.

[5] Dixon, M., *Manometric Methods*, University Press, Cambridge, 1943.

[6] Keilin, D., *Proc. Roy. Soc. Lond.*, B, 1936, **121**, 165.

[7] Spiegelman, S., *Biol. Bull.*, 1945, **89**, 122.

[8] Winzler, R. J., *Science*, 1944, **99**, 327.

being 90 minutes, data obtained from unadapted cells are given only from 90 minutes on. With fully adapted cells, only the highest inhibitor concentration was used. Table I shows a typical set of results.

Despite the fact that the O_2 consumption (not shown) was just as completely inhibited by 10^{-3} M azide in adapted as in unadapted cells, the fermentation of galactose by adapted cells was not only not inhibited, but was actually stimulated by azide. None of the other compounds used gave such results; and we therefore present the data for them somewhat more briefly. Concentration curves were obtained, but we show here only the data obtained at the highest concentrations used. The stimulatory effect of azide at low concentrations will be discussed more fully elsewhere.

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With the possible exception of fluoride, the results appear negative. In the case of IAA, however, irreversible changes tend to occur after long periods of exposure. Fluoride[9] is known to combine with magnesium and phosphate as a complex salt within the cell, this complex then uniting with the enzyme enolase: and it is not certain that this complex would have been removed by washing the cells. Consequently, the results of Table III must be regarded with some reservations.

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[9] Warburg, O., and Christian, W., *Bioch. Z.*, 1942, **310**, 384.

[10] Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1943, **21**, 77.

[11] Lundsgaard, E., *Bioch. Z.*, 1930, **217**, 162.

However, the data on this point are as yet not conclusive.

From the effects of azide in particular we conclude that adaptation to galactose fermentation involves the synthesis of one or more compounds which are not metabolic intermediates of galactose utilization, probably enzymes or coenzymes.

Summary. The effects of several enzyme inhibitors on the adaptation of yeast to galactose fermentation has been studied. The results strongly indicate that the process of adaptation involves the synthesis of one or more new enzymes.

The author wishes to thank Profs. M. B. Visscher and H. G. Wood for their support and encouragement of these studies.

15503

Influence of an Alkaline Solution on Tissue Toxicity of Uranium Nitrate.

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The observation was made in this laboratory[1] that a solution of sodium carbonate would not only protect the kidney against the toxic action of a uranium salt but that it would furthermore protect it against the injurious effect of a general anesthetic body. The first part of this observation was confirmed by Goto[2] and very recently further extended by the studies of Donnelly and Holman[3] who were able to demonstrate a similar order of protection from a solution of sodium citrate. In a later paper[4] these authors and their associates attempt an explanation for this phenomenon of protection. They attribute it not to the action of a solution of sodium citrate as an alkaline medium but they believe that their "data on sodium citrate protection against uranium injury point to the maintenance of one or more vital equilibria (possibly the 'citric acid cycle' of carbohydrate metabolism) while the normal processes of repair are taking place, for we have no evidence that repair is accelerated or altered in any way." It becomes difficult to see how these authors could come to such a conclusion without employing control experiments in which sodium carbonate[1] or sodium bicarbonate[2] were used as agents to induce protection against uranium. Such an observation is especially appropriate when the fact is well established that citrates are rapidly and completely changed in the tissues to carbonates.

The way in which an alkaline solution protects the kidney, the liver and likely other tissues against a uranium injury is not known. It is furthermore not known how uranium induces its injury. Holman and Douglas[5] have shown they were able to recover from 31 to 88% of uranium nitrate from the urine of dogs during the first 24 hours of an intoxication by 5 mg of this substance per kg of weight. It is at this period that the renal injury commences and rapidly progresses. The suggestion is here repeated[1] that the cellular toxicity of uranium salts may be due to their ability to inhibit processes of intracellular oxidation and that such an inhibition is due to the characteristic of the uranium atom to be radioactive. Such an inhibition would lead to an intracellular accumulation of hydrogen ions with a change in the chemical environment in which various oxidation reduction systems exert their

1 MacNider, Wm. deB., *J. Exp. Med.*, 1916, **23**, 171.

2 Goto, Kingo, *J. Exp. Med.*, 1917, **25**, 693.

3 Donnelly, G. L., and Holman, Russell, *J. Pharm. and Exp. Therap.*, 1942, **75**, 11.

4 Donnelly, G. L., Ross, C. J., Meroney, W. H., and Holman, Russell L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 75.

5 Holman, Russell L., and Douglas, William A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 72.

influence. The fact that such a tissue disturbance does exist is shown by the observation that a reduction in the reserve alkali of the blood invariably occurs in the dog during the course of a uranium intoxication.[6] With this order of reasoning, the suggestion is made that the intravenous use of an alkaline solution: carbonate, bicarbonate or citrate, exerts its tissue protection not by the neutralization of hydrogen ions in the sense that they are as such responsible for the tissue damage, but by maintaining through such a binding or neutralizing effect an intracellular chemical environment of such an order of balance (acid-base) that various enzymatic systems can operate in an effective manner as life processes.

[6] MacNider Wm. deB., *J. Exp. Med.*, 1917, **26**, 1.

15504

Effect of Parabiosis on Experimental Uremia.*

JOHN A. SIBLEY AND CHARLES HUGGINS.
From the Department of Surgery, University of Chicago, Chicago, Ill.

The purpose of these experiments was to study the efficiency of peritoneal anastomosis in parabiotic rats, one member of the pair being uremic.

Putnam[1] demonstrated in cats that fluids in the peritoneal cavity came into almost complete osmotic equilibrium with the blood plasma. Ganter[2] first studied peritoneal lavage as a therapeutic measure, noting that the introduction of salt solution into the peritoneal cavity of dogs with bilateral ureteral ligation improved the resulting uremic symptoms. It has been demonstrated that peritoneal lavage can reduce the values of nonprotein nitrogenous constituents of the blood[3-6] and prolong the lives of uremic dogs[7,8] and patients.[9,10] Frank, Seligman and Fine[11] eliminated uremia in a patient with acute renal failure by peritoneal irrigation.

A number of investigators[12-15] have studied renal function by the method of parabiosis. Herrmansdorfer[12] joined pairs of rats in parabiosis with coelioanastomosis and later removed the kidneys of one of the parabionts; he was able to remove 3 kidneys from the 2 rats and maintain life without a rise in nonprotein nitrogen, or other constituents of the blood. All of these investigators produced

* This study was aided by a grant from Mr. Ben May, Mobile, Alabama, from the Albert and Mary Lasker Foundation, Inc., and from the Sidney and Frances Brody Foundation.

[1] Putnam, T. J., *Am. J. Physiol.*, 1923, **63**, 548.

[2] Ganter, G., *München. med. Wchnschr.*, 1923, **70**, 1478.

[3] Landsburg, M., and Groinski, H., *C. R. Soc. de biol.*, 1925, **93**, 787.

[4] Rosenak, S., and Siwon, P., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1926, **39**, 391.

[5] Heusser, H., and Werder, H., *Bruns' Beitr. z. klin. Chir.*, 1927, **141**, 38.

[6] Curtis, G. M., and Pacheco, G. A., PROC. SOC. EXP. BIOL. AND MED., 1928, **26**, 874.

[7] Bliss, S., Kastler, A. D., and Nadler, S. B., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1078.

[8] Jeney, A. von, *Z. f. klin. Med.*, 1932, **122**, 294.

[9] Balaza, J., and Rosenak, S., *Wien. klin. Wchnschr.*, 1934, **47**, 851.

[10] Wear, J. W., Sisk, I. R., and Trinkle, A. J., *J. Urol.*, 1938, **39**, 53.

[11] Frank, H. A., Seligman, A. M., and Fine, J., *J. A. M. A.*, 1946, **130**, 703.

[12] Herrmansdorfer, A., *Deutsche Z. f. Chir.*, 1923, **178**, 289.

[13] Jeffers, W. A., Lindauer, M. A., Twaddle, P. H., and Wolferth, C. C., *Am. J. M. Sc.*, 1940, **199**, 815.

[14] Grollman, A., and Rule, C., *Am. J. Physiol.*, 1943, **138**, 587.

[15] Dannheisser, F., *Deutsche Z. f. Chir.*, 1931, **232**, 688.

the uremic state at a sufficiently long time after parabiosis for a common circulation to exist, while in the present experiments parabiotic union and uremia were established simultaneously. That an actual anastomosis of the 2 circulations eventually develops has been repeatedly shown by the passage of india ink, bacteria, avian blood cells, and dyes.[14,16-18] Studies made before the development of vascular communications are few; Spagnol[19] showed that colloidal dyes injected into one member of the pair did not cross to the mate during the first few days after anastomosis.

Experimental. Uremia was produced by bilateral nephrectomy in 8 adult albino rats and these rats died within 72 hours after operation. They became apathetic and later comatose; occasionally there was tetany. Fluid retention with massive edema and pleural fluid occurred. The nonprotein nitrogen of whole blood rose rapidly from the normal of 25 to 45 mg % to about 150 mg % in 24 hours, (Fig. 2) and to about 350 mg % before death.

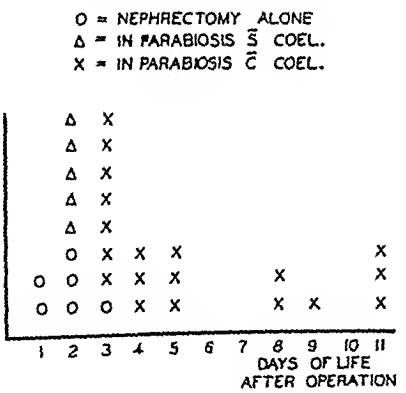

FIG. 1.
The duration of life in nephrectomized rats: after nephrectomy alone, 0; nephrectomy in a rat in parabiosis without peritoneal anastomosis, Δ; nephrectomy in one partner in parabiosis with coelio-anastomosis, X.

16 Hill, R. T., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 592.

17 Hill, R. T., *J. Exp. Zool.*, 1932, **63**, 203.

18 Hill, R. T., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 922.

19 Spagnol, G., *Arch. di fisiol.*, 1930, **28**, 157.

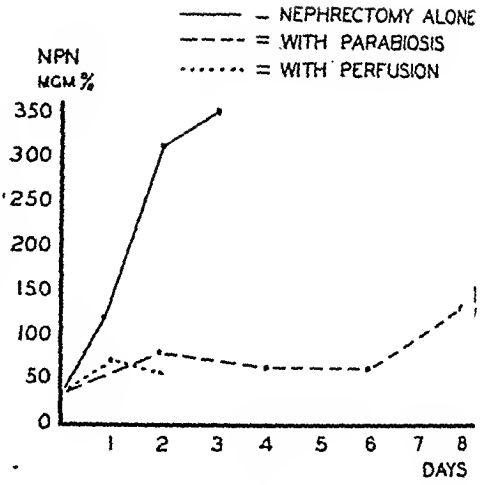

FIG. 2.
Nonprotein nitrogen of whole blood in nephrectomized rats.

The effect of parabiosis of such a nephrectomized rat with a normal rat was tried. A procedure similar to the original method of Sauerbruch and Heyde[20] was employed in which the skin of the animals was joined from neck to hip, the scapulæ were sutured together, and a large anastomosis produced between the 2 peritoneal cavities. The type of circulation obtained between the 2 animals was studied. Normal rats, injected intravenously with Evans' blue dye (T-1824) 3 cc of 1% solution, remain blue in color for many months. This amount of Evans' blue was injected intravenously in one rat, and during the first 4 days after parabiosis no transfer to the partner was observed as shown by the unchanged color of the animals and by colorimetric studies on the serum. After 4 days blue coloration was observed to extend into the skin of the uninjected rat for about 1 cm beyond the suture line. On the 6th to the 7th day the dye spread over the entire partner, and studies of the blood serum showed a nearly equal concentration of dye in the serum of both animals. It was therefore concluded that during the first 4 or 5 days the only form of communication was the common peritoneal cavity and the tissue spaces at the anastomosis.

20 Sauerbruch, F., and Heyde, M., *München. med. Wchnschr.*, 1908, **55**, 153.

Bilateral nephrectomy was performed on one of the rats of 22 pairs at the time of parabiosis. The pairs then lived for 3 to 11 days before the nephrectomized rat died. To insure that the coelio-anastomosis was of importance in maintaining life, parabiosis of a nephrectomized rat with a normal mate was done in 6 pairs without communication of the body cavities. All of these pairs died in 3 days or less, as did the single nephrectomized rats (Fig. 1).

Nonprotein nitrogen studies of whole blood showed that coelio-anastomosis was usually effective in maintaining low values, although shortly before death values of 100 to 200 mg % were sometimes reached. The nonprotein nitrogen of the nephrectomized rat usually remained between 75 and 100 mg % (Fig. 2) while that of the normal partner rose to 50 to 75 mg %.

The ability of the partner to excrete phenol red (PSP) injected into the nephrectomized rat was determined. Although there was negligible excretion in the urine when the rats were in parabiosis without coelio-anastomosis, good values were obtained with peritoneal anastomosis. After 5 mg of PSP were injected intramuscularly 0.1 to 0.2 mg was excreted by the partner in the first 6 hours. The values tended to increase when repeated after 5 days, probably due to the development of an anastomosis of the circulations.

In 15 experiments the peritoneal cavity of a single nephrectomized rat was continuously irrigated with Tyrode's solution. Although the life of the nephrectomized rat was not lengthened over that of the nephrectomized control the nonprotein nitrogen remained at values between 75 and 100 mg %. Absorption of fluid took place and death apparently was due to increased pleural fluid, and pulmonary edema.

Conclusion. These experiments demonstrated the efficiency of the peritoneal membrane as a mode of excretion in renal insufficiency. The effectiveness of the natural dialyzor provided by coelio-anastomosis suggests the feasibility of developing an artificial dialyzing mechanism.

15505 P

"Dietary Factor" in Necrotizing Arteritis in Dogs a Lipid Substance.*

RUSSELL L. HOLMAN AND MARGARET C. SWANTON.

From the Department of Pathology, University of North Carolina, Chapel Hill, and the Department of Laboratories, Watts Hospital, Durham, N.C.

During the past several years arterial lesions affecting principally the large elastic arteries (aorta, pulmonary artery, endocardium of the left auricle, and coronary arteries) have been produced with regularity by controlling 2 factors, diet and renal insufficiency.[1] The arterial lesions have been described and illustrated in previous publications, and the evidence for a dietary factor has been reviewed in a recent publication.[1]

In this paper is presented a summary of the data obtained from systematically testing the ingredients of the "standard diet" which was being fed at the time these unanticipated lesions were first encountered.

Method. The methods have been detailed in a previous publication.[1] Briefly, these consist of feeding a specified diet for a specified period of time (usually 8 weeks or longer), then damaging the kidneys (usually with heavy metal injury), and examining the arterial system both grossly and histologically when the animals die or are sac-

* This work was aided by a grant from The John and Mary R. Markle Foundation. We are indebted to Distillation Products, Inc., for the crystalline vitamin A and vitamin D used in these studies.

[1] Holman, R. L., *Am. J. Path.*, 1941, **17**, 359; *Am. J. Path.*, 1943, **19**, 977.

TABLE I.
Influence of Diet on Incidence of Arterial Lesions Following Kidney Damage.

Diet	Weeks*	No. with lesions	
		No. in group	% positive
Standard (6% C.L.O.)	8-22	26/30	86.7
Kennel	?	5/111	4.5
Kennel + C.L.O.	8-15	22/25	88.0
Standard (corn oil substituted for C.L.O.)	8-13	0/6†	0.0

* Weeks of dietary feeding before production of kidney damage.
† One dog in this group had extensive necrotizing arteriolitis.

rificed (usually days or weeks after the renal injury).

"Standard diet," the diet that was being fed at the time these unanticipated lesions were first encountered, consisted of calves' liver (raw wet weight), 32 parts; cane sugar, 25 parts; corn starch, 25 parts; butter, 12 parts; and commercial cod liver oil (USP XI—vitamin A, 850 I.U. per g, and vitamin D, 85 I.U. per g), 6 parts. Enough tomato juice was added to make a paste of which each gram contained 3 calories. The diet was fed in amounts to furnish 75 calories per kg per day. Five grams of kaolin and 1 g of salt mixture were added to each day's diet. Essentially, this is a low protein diet with only 7% of its caloric value derived from protein, 43% from fat, and 50% from carbohydrate. With the "alterations" in this diet, *i.e.*, doubling, halving, or omitting different ingredients of the diet, care was taken to keep it isocaloric.

"Kennel diet," unless otherwise specified, consisted of selected table scraps from the University dining halls.

Both of these diets were kept in a refrigerated room (38-40°F) and were fed in a reasonably fresh condition. In other words, there was no reason to suspect rancidity or other forms of spoilage.

Renal injury was usually produced by minimum lethal dosage of heavy metal which, in the case of uranium nitrate, consisted of 5.0 mg of uranyl nitrate per kg of body weight injected subcutaneously in the hypochondrium in 0.5% aqueous solution, and in the case of mercuric chloride, consisted of 3.0 mg per kg body weight injected intravenously in one of the external jugular veins in 0.1% aqueous solution.

Results. In this preliminary report the detailed data obtained from doubling and halving the various ingredients of the standard diet are omitted, and all of the pertinent data to date are combined in one simplified table (Table I). Suffice it to say that all the negative as well as all the positive data that have been obtained point to something of lipid nature that (in dogs at least) has to be fed for a period of 8 weeks or longer before experimentally-induced kidney damage is regularly followed by arterial lesions.

Analysis of the data in Table I yields 3 types of evidence: (1) definite evidence for a dietary factor; (2) evidence that the dietary factor is contained in commercial cod liver oil; and (3) evidence that the dietary factor is not unique to cod liver oil—the finding of typical arterial lesions in 5 control dogs, *i.e.* dogs fed only kennel diet for an indefinite period before their kidneys were damaged, all occurred during an 18-month period when kennel diet consisted of beef bones with much adherent fat;[2] and the dog fed corn oil instead of cod liver oil that had extensive necrotizing arteriolitis is additional evidence that the dietary factor is not unique to cod liver oil. All the data implicate a substance (or substances) of lipid nature. The dietary factor is heat stable, is not readily oxidized, is not vitamin A, and is not vitamin D. Studies designed to further identify the dietary factor are in progress.

Comment. There is one important difference between these studies and previous

[2] Holman, R. L., *J. Exp. Med.*, 1945, **81**, 399.

ones[3] on arterial lesions related to cod liver oil—*renal insufficiency*. The manner in which renal insufficiency is produced (heavy metal injury, bilateral nephrectomy,[4] *Leptospira canicola*[5] is relatively unimportant, but some degree of renal damage is *essential*. The "standard diet" or kennel diet plus cod liver oil can be fed to dogs indefinitely (at least as long as a year) and no arterial lesions are ever observed until renal function is disturbed. This emphasizes the role of the kidney in the internal metabolism of the "dietary factor," which presumably is lipid in nature.

As time goes by, we gain confidence that arterial lesions can be produced with regularity in dogs by controlling 2 factors, (1) diet and (2) kidney damage; that both factors are necessary, and that only these 2 factors are involved.

[3] Agduhr, E., and Senstrom, N., *The Appearance of the Electrocardiogram in Heart Lesions Produced by Cod Liver Oil Treatment*, Uppsala, Almguist and Wiksells, 1930; Cowdry, E. V., *Arteriosclerosis*, New York, The Macmillan Co., 1933.

[4] Holman, R. L., *Am. J. Path.*, 1943, **19**, 159.

[5] Holman, R. L., uupublished data.

15506

Pigment Studies on the Incisor Teeth of Vitamin E Deficient Rats of the Long-Evans Strain.*

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Several investigators[1-5] have reported that vitamin E deficient rats, over a period from 45 to 222 days, lose their natural brownish yellow pigment of the maxillary incisor teeth. During the past 9 years we have had occasion to examine several hundred vitamin E deficient rats of the Long-Evans strain. Although we did not check specifically for abnormal white incisors, we feel reasonably sure that such an obvious change in the pigmentation of the teeth would not have passed entirely unnoticed. To check accurately this depigmentation phenomenon in our strain of rats, the following experiment was conducted.

Procedure. Four groups (Groups 3, 5, 9, 11) of rats were maintained on our vitamin E deficient Diet No. 5† for either 310 or 460 days. Other groups (Groups 1, 7) were given Mason's vitamin E deficient Diet No. 69‡ for 130 days. Each of these groups was compared with normal control groups fed a commercial dog biscuit diet plus bi-

* Aided by a graut from the Milbank Memorial Fund of New York. The following materials were generously eontributed: brewers' yeast by The Vitamin Food Company of Newark, N.J., and the eod liver oil by E. R. Squibb and Sons of New York City.

1 Dam, H., and Granados, H., *Science*, 1945, **102**, 327.

2 Davis, A. W., and Moore, T., *Nature*, 1941, **147**, 794.

3 Granados, H., and Dam, H., *Science*, 1945, **101**, 250.

4 Granados, H., and Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 295.

5 Moore, T., *Biochem. J.*, 1943, **37**, 112.

† Diet No. 5:

Casein (commercial)	24.0
Cornstarch (uncooked)	35.0
Salts No. 2	5.0
Lard	20.0
Cod Liver Oil	2.0
Brewers' Yeast	10.0
Cellulose Flour	4.0

All diet ingredients, except the eod liver oil, were mixed and then allowed to stand at room temperature for 2 weeks. The eod liver oil was added just before feeding.

TABLE I.

Maxillary Incisor Pigment Observations in Vitamin E-Deficient Rats.

(All rats were placed on the experimental diets at 21 days of age. The figures in the parenthesis represented the range of pigment values within each group. The unit 10 represented the maximum amount of normal pigmentation. Variations of the groups considered significant if P = 0.05 or less.)

Group	Diet	Days on diet	No. in group	Mean degree of pigmentation	S.E.	P.
		Females.				
1	E-Deficient No. 69	130	14	6.4 (6-9)	0.26	>0.05
2	Normal	130	12	7.0 (6-8)	0.23	
3	E-Deficient No. 5	310	18	7.3 (6-10)	0.13	<0.01
4	Normal	310	12	8.5 (6-10)	0.17	
5	E-Deficient No. 5	460	8	6.8 (6-8)	0.14	<0.01
6	Normal	460	7	8.4 (6-10)	0.27	
		Males				
7	E-Deficient No. 69	130	11	7.0 (5-8)	0.27	>0.05
8	Normal	130	13	7.6 (5-8)	0.21	
9	E-Deficient No. 5	310	15	7.5 (6-9)	0.11	>0.1
10	Normal	310	10	7.8 (5-10)	0.19	
11	E-Deficient No. 5	460	4	7.2 (5-9)	0.27	>0.1
12	Normal	460	5	7.6 (6-8)	0.14	

weekly supplements of fresh lettuce. During the last 60 days of the experiment the amount of pigmentation was carefully estimated with the use of a color chart with values 1 to 10. The value 10 representing an incisor tooth with a dark brown pigmentation, and the value 1 representing a chalky white incisor devoid of any pigment.

Results. The male rats in all groups at the termination of the experiment had almost identically the same degree of pigmentation in the maxillary incisors regardless of the diet used. The female rats, however, did show some slight variation. In the deficient animals the upper incisors were slightly less pigmented than in the control group. The largest variation was found in Groups 3 and 5 (Table I). The maxillary incisors of the control group had an average reading of 8.5, 8.4 as compared to 7.3, 6.8 of the deficient animals respectively. This is a significant difference but does not remotely approach the almost complete depigmentation in E deficient albino rats held on the vitamin E free diet for only 45 days as reported by Granados and Dam.[3]

The mandibular incisors in the deficient animals of both sexes uniformly had slightly higher pigmentation readings than the control rats. The greatest difference was in the 310-day-old male animals (Groups 9, 10). The deficient male group had a reading of 3.4 while the male control group had only a 2.3 reading. These were not sig-

‡ Mason's Diet No. 69:

Casein (commercial)	20.0
Cornstarch (uncooked)	50.0
Salts (Hubbell's)	2.5
Lard	18.0
Brewers' Yeast	7.5
Cod Liver Oil	2.0

nificant differences.

Discussion. It was obvious that our strain of rats was not showing the striking depigmentation phenomenon reported by other investigators. Several factors were investigated for an explanation for our findings. At the onset of this experiment we felt that the type and amount of iron used in the different diets might account for the discrepancies, since ferric iron is the cause of the pigment color.[1,4,5] Our Diet No. 5 contained 155 mg of ferric alum citrate per 100 g of diet. Mason's Diet No. 69 contained 50 mg of ferric phosphate per 100 g of diet. Since the rats on the latter diet (Groups 1, 7) failed to develop any significant depigmentation, we concluded that neither the differences in the iron compounds nor the unequal amounts of iron consumed by the rats were the determining factors in the failure of our rats to manifest any significant depigmentation of their incisors.

The presence of highly unsaturated fatty acids in the diet is a predisposing factor for depigmentation.[1] Both of the deficient diets, however, had equal amounts of cod liver oil and only slight differences in lard content (18-20%). Diet No. 5, however, had a shelf age of 2 weeks and was, therefore, slightly rancid upon feeding. In contrast, Diet No. 69 contained only fresh lard as it was prepared weekly and fed immediately to the animals. The fluctuation in the amounts of unsaturated fatty acids due to the incipient rancidity in Diet No. 5 is probably negligible. We believe, therefore, that the failure of our vitamin E deficient rats to show marked depigmentation of the maxillary incisors is not due to any obvious diet differences. Age and sex differences in our animals and those of other workers were not determining factors since our observations were on both sexes and the duration of our experiment equaled or exceeded that of the previous investigators.

The factor responsible for this disagreement with other workers on this problem is probably due to the difference in strain of our animals. All investigators, except one, reporting this peculiar depigmentation used albino rats. (Moore[5] used both albino and piebald rats). Our rats were inbred hybrids. The strain was started by Dr. Long in 1908 from a wild male grey rat and 2 tame albino rats.[6] The inbreeding of this hybrid strain may be the determining factor as it seems to be the only important variable not already accounted for. While no data are available on the effect of vitamin E deficiency on the wild grey rat, a recent note from Granados, *et al.*,[7] claimed that Syrian hamsters showed a greatly delayed depigmentation pattern as compared to the albino rat. They also reported that the Florida cotton rat showed no loss of incisor pigment after 150-180 days on an E-deficient diet. Thus this latter "wild" rat reacted to the deficiency in much the same manner as did our hybrid "wild" strain rats. It might be possible that these "wild" strains of rodents have the capacity to either manufacture or retain greater amounts of this tooth pigment than do the long established domesticated strains. Furthermore, Irving[8] also reported no depigmentation in the incisors of 3 female rats after 167 days on an E-deficient diet. Unfortunately, however, he did not indicate the strain of rat used.

Conclusions. 1. Vitamin E deficient rats of the Long-Evans strain did not show the extensive depigmentation of the maxillary incisors as reported by the other investigators. 2. Female rats maintained on an E-deficient diet for 310 days or longer gradually developed a slight incisor depigmentation that was statistically significant. Male rats on similar diets failed to manifest a significant change. 3. A comparison of diet differences, sex and age variations in our animals and those of other workers failed to provide an adequate explanation for our contradictory results. 4. It is suggested that a genetic difference between the Long-Evans and albino strains of rats may be the explanation for the failure of the former strain to show the maxillary incisor depigmentation earlier reported in vitamin E deficient albino rats.

[6] Long, J. A., personal communication.

[7] Granados, H., Mason, K. E., and Dam, H. (Proc. 1946 Meeting International Association for Dental Research), *J. Dental Research*, in press.

[8] Irving, J. T., *Nature*, 1942, **150**, 122.

15507

Gonadotrophic Hormone Secretion in Immature Hypophysectomized Parabiotic Rats.*

CLYDE BIDDULPH AND ROLAND K. MEYER.

From the Department of Zoology, University of Wisconsin, Madison.

Following gonadectomy of one parabiotic rat of adult pairs, the other partner develops constant vaginal estrus if it is a female.[1,2] Likewise constant estrus appears in the hypophysectomized partner of adult hypophysectomized parabiotic rats following gonadectomy of the other parabiont.[2-5] In order to gain information concerning the kind and relative amount of gonadotrophic hormone released into the blood following gonadectomy in immature rats, experiments were designed in which parabiotic rats of both sexes were hypophysectomized and at the same time their parabiotic mates were gonadectomized. The gonad response of the hypophysectomized partner was used as the end-point in determining the nature of the pituitary secretion.

Materials and Methods. Littermate rats weighing 70 g or more were united in parabiosis at 31 to 33 days of age. The operative technic was that described by Bunster and Meyer,[6] except that metal skin clips were used instead of silk sutures in closing the skin incisions. On the 6th day after the animals were united, the left partner was hypophysectomized and the right partner was gonadectomized. All operations were performed under sterile conditions and under ether anaesthesia.

The majority of the animals were autopsied on the 11th day following the hypophysectomy-gonadectomy operation. The remainder were killed between the 3rd and 53rd days following this operation. At autopsy the gonads and accessories were removed and weighed. The ovaries were examined by the use of transmitted light to determine the qualitative response and the sella turcica was examined under binoculars and checked with the operation notes for completeness of the hypophysectomy.

Results and Discussion. The data reported in Table I give the weight and qualitative response of the ovaries of the hypophysectomized female following gonadectomy of its female or male partner. It will be noted that within 3 days following gonadectomy sufficient gonadotrophic hormone appeared in

TABLE I.
Ovarian Response of Hypophysectomized-Castrated Parabiotic Rats.

Sex of pairs	No. days hypophysectomized	Hypophysectomized partner: Ovarian wt, mg*	Hypophysectomized partner: Qualitative response
♀ ♀	3	29	Follicles
	5	26	"
	6	32	"
	7	26	"
	7	33	"
	7	15	"
	9	24	"
	9	36	"
	10	32	"
	10	11	"
	13	13	"
	17	11	"
♀ ♂	4	26	"
	8	31	"
	10	50	"
	10	23	"
	10	37	"
	10	40	"
	10	30	"
	53	59	"

* The average ovarian weight of single hypophysectomized control rats after being hypophysectomized for 10 days was 4.0 mg.

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

1 Hill, R. T., *J. Exp. Zool.*, 1932, **63**, 203.

2 Meyer, R. K., and Biddulph, C., *Am. J. Physiol.*, 1941, **134**, 141.

3 Witschi, E., and Levine, W. T., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 101.

4 Möller-Christensen, E., *Acta Path. et Microbiol. Scand.* (Suppl.), 1935, **22**, 1.

5 DuShane, G. P., Levine, W. T., Pfeiffer, C. A., and Witschi, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 339.

6 Bunster, E., and Meyer, R. K., *Anat. Rec.*, 1933, **57**, 339.

the bloodstream to give a substantial ovarian response in the hypophysectomized female. This indicates that the pituitary gland increases its output of gonadotrophic hormone very soon after the gonads are removed. Regardless of the sex of the gonadectomized animal follicles only were present in the ovaries of the hypophysectomized female, at least for a period of 53 days following gonad removal. From this evidence it follows that FSH was released from the anterior pituitary gland of the gonadectomized rat and that it passed via the circulation to the hypophysectomized parabiont where the ovaries were either maintained at their pre-hypophysectomy level or were stimulated to develop follicles and to increase in size.

The question arose as to whether or not the ovaries contained only follicles because FSH was the only gonadotrophic hormone released from the pituitary gland or whether this was due to a refractory or unresponsive state of the ovaries of the hypophysectomized partner to LH. That the former is more likely true is indicated by the presence of corpora lutea in the ovaries of the hypophysectomized partner of one pair (not included in Table I) in which a fragment of pituitary gland remained in the sella turcica at the time of autopsy, the supposition being that the LH producing the luteinization came from this pituitary fragment. That the ovaries of the hypophysectomized partner were not refractory to LH is indicated by the results of experiments reported previously in adult hypophysectomized pairs[2] and in the accompanying paper,[7] which show that LH injected into a normal or a gonadectomized parabiont produces luteinization in the ovaries of its hypophysectomized mate.

From the above evidence it appears that LH is not released from the pituitary gland of a gonadectomized parabiotic rat during the early stages of castration, or if it is released, the concentration does not reach a sufficiently high level in the nongonadectomized partner to produce luteinization of the stimulated follicles of that rat. It would seem then that the luteinization of the ovaries of the intact parabiont which follows gonadectomy of the other rat, which has been reported in previous papers from this laboratory[8-11] and by others, is produced by LH from the pituitary gland of the intact rat. Evidently the stimulus effecting release of LH is the estrogen produced by the large stimulated ovaries of the intact rat, the FSH originating from the pituitary gland of the gonadectomized rat. However, it should be noted that the injection of estrogen into adult parabiotic rats does not cause the release of LH from the castrate pituitary gland.[2]

Hellbaum and Greep[12] have reported that the pituitary gland of normal adult female rats contains relatively large amounts of LH, and that of normal males is relatively deficient in this factor. Following castration the presence of LH in the pituitary of male rats becomes definitely evident about 20 days after gonadectomy as judged by assay of their pituitary glands. One of our female-male pairs was allowed to live for 53 days, but during this time only follicular stimulation was evident in the ovaries, which indicates that LH was not released by the pituitary gland, or if it was released the concentration did not reach a high enough level to produce luteinization.

Table II presents the results obtained when the hypophysectomized and gonadectomized parabionts were both males. It will be observed that there is considerable variation among the pairs in the weight of the testes, seminal vesicles and prostate of the hypophysectomized partner. Comparison of average weights of these structures with the corresponding average weights of normal animals indicates that there was some decline

[7] Meyer, R. K., Biddulph, C., and McShan, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 95.

[8] Meyer, R. K., and Hertz, R., *Am. J. Physiol.*, 1937, **120**, 232.

[9] Hertz, R., and Meyer, R. K., *Endocrinology*, 1937, **21**, 756.

[10] Biddulph, C., Meyer, R. K., and Gumbreck, L. G., *Endocrinology*, 1940, **26**, 280.

[11] Biddulph, C., Meyer, R. K., and Gumbreck, L. G., *J. Exp. Zool.*, 1941, **88**, 17.

[12] Hellbaum, A. A., and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 902.

15507

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	9	24	"
	9	36	"
	10	32	"
	10	11	"
	13	13	"
	17	11	"
♀♂	4	26	"
	8	31	"
	10	50	"
	10	23	"
	10	37	"
	10	40	"
	10	30	"
	53	59	"

* The average ovarian weight of single hypophysectomized control rats after being hypophysectomized for 10 days was 4.0 mg.

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Table II presents the results obtained when the hypophysectomized and gonadectomized parabionts were both males. It will be observed that there is considerable variation among the pairs in the weight of the testes, seminal vesicles and prostate of the hypophysectomized partner. Comparison of average weights of these structures with the corresponding average weights of normal animals indicates that there was some decline

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[8] Meyer, R. K., and Hertz, R., *Am. J. Physiol.*, 1937, **120**, 232.

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[12] Hellbaum, A. A., and Greep, R. O., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 902.

TABLE II.
Weights Obtained in Hypophysectomized-Castrated Male Parabiotic Rats.

Sex of pairs	No. days hypophysectomized	Hypophysectomized partner* (mg)				Castrated partner (mg)		
		Testes	Sem. Vesic.	Prost.	Adren.†	Sem. Vesic.	Prost.	Adren.
♂ ♂	10	1733	130	177	17	10	22	28
	10	1760	92	122	12	8	8	30
	10	1212	20	51	19	7	17	39
	10	1438	162	172	20	12	26	25
	10	1516	129	144	24	9	27	26
	10	1900	251	166	15	12	19	30
	10	382	8	22	13	7	15	25
	10	1611	25	64	15	10	12	29
	10	1256	185	178	12	10	13	32
	10	1538	164	175	20	—	—	35
	10	1943	280	284	17	7	21	34
	10	1885	160	282	16	13	25	32
	10	1357	14	32	14	13	34	33
	11	645	13	22	16	8	14	39
	11	2141	375	313	12	9	16	25
	Averages	1474	133	147	16	9	18	31

* The testis, seminal vesicle and prostate weights of 7 normal animals of the same age were 2042, 101, and 170 mg, respectively

† The average adrenal weight of single hypophysectomized control rats of the same age was 10 mg.

in testis weight of the hypophysectomized partner, but that the accessory weights of this rat were approximately the same as those of normal controls. Usually when there was a marked decline in testis weight in a given rat the accessory gland weights likewise declined, which shows that the endocrine function of the testes decreases when the testes are not maintained by gonadotrophic hormones. It should be emphasized that in every case the testes of the hypophysectomized partner, regardless of their size and the size of the accessories, remained in the scrotum throughout the experiment. This is in agreement with the results of other workers.

The data obtained when the hypophysectomized partner was a female indicates that only FSH passed from the castrated male to the hypophysectomized partner. From the fact that neither the testes nor the accessories of the hypophysectomized male underwent the typical regressive changes of hypophysectomy, it is suggested that (1) endogenous FSH in the amounts present following gonad removal is concerned with the maintenance of the testes in the scrotum, for it seems logical to assume that FSH also passed from the gonadectomized male to the hypophysectomized male in these experiments, or (2) that small amounts of LH were also released from the pituitary gland following gonadectomy of the male and that these small amounts were sufficient to stimulate the interstitial cells of the testes to produce androgen, but were not enough to produce luteinization of the ovaries. The experiments reported in this paper on female-male pairs confirm the results of Greep[13] on parabiotic triplets which demonstrated that the gonadotrophins secreted following gonadectomy of a male produce only follicles in the ovary and stimulate the testes and accessory glands of the male.

Cutuly, *et al.*[14] have reported experiments with adult hypophysectomized-castrated male parabiotic rats in which varying degrees of gonadal and accessory maintenance in the hypophysectomized partner were obtained. Our results in immature animals are essentially the same as those reported by

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The variation in ovarian, testis and accessory weights in the hypophysectomized partner may be a reflection of the degree to which the pituitary gland undergoes castration changes following gonad removal. We have previously reported[11] that approximately 11% of a large number of control parabiotic rats failed to undergo the typical ovarian enlargement following gonadectomy of one parabiont. The percentage of rats failing to show the typical castration changes in this study when the recipient parabiont was hypophysectomized is slightly higher (17%), which indicates that the gonads of hypophysectomized rats respond less readily to gonadotrophic stimulation than those of normal rats.

Summary. Using immature hypophysectomized-gonadectomized parabiotic rats it has been demonstrated that following gonad removal in both sexes follicular stimulation only is obtained in the ovaries of the hypophysectomized parabiont for as long as 53 days following the operation. If the hypophysectomized partner is a male, the testes are maintained in the scrotum and their average weight is somewhat less than that of normals, whereas the weight of the accessory glands is approximately the same as that of normal animals.

Approximately 17% of the pairs failed to show any gonadal stimulation following the hypophysectomy-gonadectomy procedure. This percentage failure is slightly greater than that occurring in nonhypophysectomized pairs.

15508

Luteinization of the Ovaries of Immature Hypophysectomized Parabiotic Rats with Gonadotrophic Hormone Preparations.*

ROLAND K. MEYER, CLYDE BIDDULPH, AND W. H. MCSHAN.

From the Department of Zoology, University of Wisconsin, Madison.

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Materials and Methods. Littermate rats weighing 70 g or more were united in parabiosis at 31 to 33 days of age. The operative technic was that of Bunster and Meyer[3] except that metal skin clips were used instead of silk sutures in closing the skin incisions. On the 6th day following parabiotic union of the animals one partner was hypophysectomized and the other was either gonadectomized or left intact. All operations were performed under sterile conditions and under ether anesthesia.

Within a few hours after the operation,

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TABLE II.
Weights Obtained in Hypophysectomized-Castrated Male Parabiotic Rats.

Sex of pairs	No. days hypophysectomized	Hypophysectomized partner* (mg)				Castrated partner (mg)		
		Testes	Sem. Vesic.	Prost.	Adren.†	Sem. Vesic.	Prost.	Adren.
♂ ♂	10	1733	130	177	17	10	22	28
	10	1760	92	122	12	8	8	30
	10	1212	20	51	19	7	17	39
	10	1438	162	172	20	12	26	25
	10	1516	129	144	24	9	27	26
	10	1900	251	166	15	12	19	30
	10	382	8	22	13	7	15	25
	10	1611	25	64	15	10	12	29
	10	1256	185	178	12	10	13	32
	10	1538	164	175	20	—	—	35
	10	1943	280	284	17	7	21	34
	10	1885	160	282	16	13	25	32
	10	1357	14	32	14	13	34	33
	11	645	13	22	16	8	14	39
	11	2141	375	313	12	9	16	25
	Averages	1474	133	147	16	9	18	31

* The testis, seminal vesicle and prostate weights of 7 normal animals of the same age were 2042, 101, and 170 mg, respectively

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in testis weight of the hypophysectomized partner, but that the accessory weights of this rat were approximately the same as those of normal controls. Usually when there was a marked decline in testis weight in a given rat the accessory gland weights likewise declined, which shows that the endocrine function of the testes decreases when the testes are not maintained by gonadotrophic hormones. It should be emphasized that in every case the testes of the hypophysectomized partner, regardless of their size and the size of the accessories, remained in the scrotum throughout the experiment. This is in agreement with the results of other workers.

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Injection of Gonadotrophic Extracts into Hypophysectomized Parabiotic Rats.

Condition of pair		Right partner			Left partner*	
Left	Right	Material injected	Days inj.	Sex of pairs	Ovarian wt, mg	Qual. response
Hypophysectomized	Gonadectomized	100 mg eq. per day LH 114	3	♀ ♂	24	Follicles
			5	♀ ♂	25	"
			7	♀ ♂	39	"
			7	♀ ♂	46	Foll. and C. L.
			10	♀ ♂	21	Follicles
			10	♀ ♂	37	"
			10	♀ ♂	7	No. stim.
			10	♀ ♀	9	" "
			10	♀ ♀	7	" "
		100 mg eq. per day LH 54	10	♀ ♀	97	C. L.
			10	♀ ♀	64	" "
			10	♀ ♀	77	" "
			10	♀ ♀	48	" "
	Normal	1.2 cc eq. per day pregnancy urine prep.	8	♀ ♀	48	" "
			10	♀ ♀	40	" "
			10	♀ ♀	46	" "
			10	♀ ♂	50	" "
	Gonadectomized	1.2 cc eq. per day pregnancy urine prep.	6	♀ ♂	112	" "
			8	♀ ♂	133	" "
			10	♀ ♂	185	" "
			10	♀ ♂	179	" "
			10	♀ ♀	117	" "
			10	♀ ♀	103	" "
			10	♀ ♀	153	" "
	Normal	50 mg eq. per day unfract. sheep pit.	3	♀ ♂	41	Follicles
			3	♀ ♂	30	"
			3	♀ ♂	30	"
			3	♀ ♀	35	"
			4	♀ ♀	30	"
			7	♀ ♂	37	Foll. and C. L.
			10	♀ ♀	74	C. L.
			10	♀ ♀	78	" "
			10	♀ ♂	99	" "
			10	♀ ♂	55	" "
			10	♀ ♂	14	" "
			10	♀ ♂	49	" "
			10	♀ ♂	60	" "
		100 mg eq. per day unfract. sheep pit.	10	♀ ♀	157	" "

* The average adrenal weight of the hypophysectomized partner was 17 mg; that of the other parabiont was 32 mg.

injection of either LH, gonadotrophin prepared from the urine of pregnant women, or unfractionated (FSH and LH)) sheep pituitary extracts was begun. Five-tenths cc of aqueous solution of the preparations was injected subcutaneously once daily into the gonadectomized or normal parabiont for 3 to 10 days following the hypophysectomy and gonadectomy. The various doses used are recorded in Table I.

At autopsy the ovaries of the hypophysectomized partner were removed and examined for their qualitative response and weighed. The adrenals of each partner were also dissected and weighed.

Results and Discussion. The results of the gonadotrophic hormone injections are found in Table I. It will be seen that both male and female rats were gonadectomized or left intact and injected with the various

preparations, the hypophysectomized partner always being a female. No difference is apparent in the results obtained with each preparation regardless of whether the gonadectomized or intact partner was a male or female.

The injection of LH54 produced an abundance of corpora lutea in each rat and the ovaries were heavier than those obtained with LH114. The latter preparation was prepared by a different method, and corpora lutea were found in the ovaries of only one animal, probably because of the low luteinizing activity of the preparation.

Injection of the pregnancy urine preparation into the normal rat of hypophysectomized-normal pairs produced corpora lutea in the ovaries of the hypophysectomized rat in all cases, the average ovarian weight being 41 mg. When the same preparation was injected into the gonadectomized partner of hypophysectomized-gonadectomized pairs it was found that corpora lutea were formed in the ovaries of the hypophysectomized rat in every instance, and the ovarian weight was much greater than before, the average being 140 mg.

The response obtained with the pregnancy urine preparation is an interesting one, for reports in the literature indicate that this type of gonadotrophin is by itself ineffective, or relatively so in hypophysectomized rats.[4-6] Regardless of whether the injected rat was normal or gonadectomized the pregnancy urine gonadotrophin used in this study luteinized the ovaries of the hypophysectomized rat.

The greater weight of the ovaries of the hypophysectomized-gonadectomized pairs was undoubtedly due to the greater stimulation of the ovaries by the combination of endogenous gonadotrophin from the gonadectomized rat's pituitary gland and injected gonadotrophin. It seems probable that the follicles present in the ovaries of the hypophysectomized partner of both types of pairs did not undergo involution immediately following hypophysectomy, and since the injections of the pregnancy urine preparation were begun immediately after the operation, the follicles became luteinized. In addition, the ovaries of the hypophysectomized partner of the hypophysectomized-gonadectomized pairs were apparently stimulated to produce new follicles by FSH from the pituitary gland of the gonadectomized partner.[1] The LH of the injected preparation acted on these stimulated follicles to produce luteinization, and consequently a greater ovarian weight was found in these pairs.

The injection of the unfractionated sheep pituitary preparation also produced luteinization of the ovaries of the hypophysectomized parabiont. In this group of animals the injections were made into the normal parabiont. A greater ovarian weight was obtained in the hypophysectomized rat with a high dose of the preparation than with a low dose.

Of interest is the fact that the adrenals of the hypophysectomized rat were partially maintained (weight 17 mg) by the adrenotrophic hormone from the nonhypophysectomized partner (adrenal weight 32 mg). The average adrenal weight of single hypophysectomized control rats of the same age was 10 mg.

From the above data it would seem that the presence of only follicular stimulation in the ovaries of the hypophysectomized-gonadectomized parabiotic rats in the previous study,[1] is due to the fact that the gonadotrophin secreted in the early stages of gonadectomy is largely FSH, little or no LH being secreted. Furthermore, the ovaries of the hypophysectomized parabiont are responsive to LH if sufficient quantities are present.

Summary. The injection of LH, gonadotrophin prepared from the urine of pregnant women, and unfractionated sheep pituitary extracts into the nonhypophysectomized partner produced luteinization in the ovaries of the hypophysectomized partner of either hy-

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pophysectomized-gonadectomized or hypophysectomized-normal parabiotic rats.

The results indicate that the failure of the ovaries of the hypophysectomized partner of hypophysectomized-gonadectomized pairs to develop corpora lutea is due to an insufficient secretion of LH following gonadectomy rather than to an unresponsive state of the ovaries of the hypophysectomized animal.

15509 P

Feather Growth Rates in Thyroidectomized Hens Following Administration of Thyroxin.*

BEN B. BLIVAISS. (Introduced by L. V. Domm.)

From the Whitman Laboratory of Experimental Zoology, The University of Chicago.

The feather growth rates of normal fowl have been shown to be modified by the administration of thyroid[1-3] as well as by estrogens.[4-6] Although a general retardation in feather regeneration has been noted in spontaneous[7] and experimental[8,9] hypothyroid conditions in fowl, no measurements were previously made on feather growth in the various body areas. Consequently we were interested in studying the effects of thyroid removal on feather growth in brown Leghorn hens and to see if thyroxin administration had the same effect on growth rates in athyroidic hens as follows its administration in normal individuals.

For the study of feather growth rates, 25 papillae in 5 rows of 5 papillae each were selected and marked in comparable areas in each bird for each of the regions measured. Measurements were made at 2- or 4-day intervals on posterior breast, anterior breast, back and saddle. All individuals studied were adults and beyond 6 months of age.

Regenerating feathers of adult thyroidectomized females, operated on or before 10 days of age, did not become measurable until 12 to 14 days after plucking as compared to 9 or 10 days in normals. During the period of rapid growth, the breast and back feathers grew more rapidly than the saddle, as seen in their growth rates and lengths on the 48th day (Table I). This is in marked contrast to the order of growth rates in normal hens where posterior breast and saddle feathers grow more rapidly than back and anterior breast. Feather growth continued for a longer period in thyroidectomized hens than in normals. Breast and back feathers revealed a lesser reduction in length and growth rate than the saddle.

In view of the well-known feather growth-promoting action of thyroid preparations in normal fowl, it seemed advisable to study the effects of various doses of thyroxin on the slow-growing feathers of athyroidic hens.

Injection of 0.5 mg of thyroxin was followed by an increase in feather growth rates within 24 hours which persisted for 12 to 16 days (Table II). The maximum growth was attained between the 2nd and 4th days after injection when growth rates attained their normal level. With this as well as higher

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. Grateful acknowledgment is made to Dr. L. V. Domm for his suggestions and interest during the prosecution of this investigation.

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2 Domm, L. V., *Anat. Rec.*, 1929, **44** (Suppl.), 227.

3 Juhn, M., and Barnes, B. O., *Am. J. Physiol.*, 1931, **98**, 463.

4 Domm, L. V., *J. Exp. Zool.*, 1927, **48**, 31.

5 Domm, L. V., and Gustavson, R. G., *Anat. Rec.*, 1929, **44** (Suppl.), 228.

6 Juhn, M., Faulkner, G. H., and Gustavson, R. G., *J. Exp. Zool.*, 1931, **58**, 69.

7 Landauer, W., *Am. J. Anat.*, 1929, **43**, 1.

8 Parhon, C. F., *C. R. Soc. Biol.*, 1924, **91**, 765.

9 Greenwood, A. W., and Blyth, J. S. S., *Proc. Roy. Soc. Edin.*, 1929, **49** 313.

TABLE I.
Feather Growth Measurements (in mm).

Ftr. age days	2 normal females				6 complete thyroidectomized females			
	Post. br.	Ant. br.	Back	Sad.	Post. br.	Ant. br.	Back	Sad.
				Average length.				
12	5.3	5.4	6.5	6.3	<1.0	<1.0	<1.0	<1.0
24	32.4	29.1	33.3	31.6	9.8	8.3	8.5	7.8
36	57.5	55.5	57.9	57.1	22.2	20.4	21.3	17.5
48	77.1	69.0	73.9	77.3	33.9	30.3	32.7	26.1
60	87.2	71.7	77.3	87.0	45.0	38.0	42.5	33.1
72	89.9	71.7	77.5	87.9	52.7	41.2	50.6	36.7
80					54.9	42.1	53.9	37.5
				2-day growth increments.				
12-24	4.5	3.9	4.5	4.2	1.6	1.4	1.4	1.3
24-36	4.2	4.4	4.1	4.2	2.1	2.0	2.1	1.6
36-48	3.3	2.2	2.7	3.4	1.9	1.7	1.9	1.4
48-60	1.7	0.4	0.6	1.6	1.8	1.3	1.6	1.2

TABLE II.
Modifications in Feather Growth Rates After Thyroxin Administration in Thyroidectomized Hens.

Dose, mg	Days inj. A	Ftr. areas	Two-day growth rate increments in millimeters Days measured post injection			
0.5	42		R-6-0	0-4	2-4	0-12
			A-36-42	42-48	44-46	42-54
		Ant. br.	1.7	3.7	4.4	3.0
		Back	1.6	3.0	3.8	3.0
		Sad.	1.7	3.1	3.7	3.1
1.0	19,		R-6-0	0-6	6-14	14-20
	26,		A-13-19	19-25	25-33	33-39
	33	Post. br.	2.3	3.8	3.9	4.9
		Ant. br.	2.2	2.7	5.2	5.1
		Back	1.9	3.6	4.0	3.8
		Sad.	1.0	2.5	4.0	4.6
1.5	18,		R-4-0	0-6	6-12	12-18
	24,		A-14-18	18-24	24-30	30-36
	30	Post. br.	2.2	4.2	5.5	5.0
		Ant. br.	2.0	2.7	3.7	4.5
		Back	2.3	3.4	5.7	3.4
		Sad.	2.2	3.8	4.8	5.8

R = Days of measurement in relation to injection time.
A = Actual days of measurement based on feather age.
Figures represent average of right and left feather areas in all cases.

doses, the increased growth rate persisted for a longer period in the saddle than in the other feather areas, indicating a lower threshold for saddle feathers.

In an attempt to obtain normal growth rates for a longer period, larger doses of thyroxin were administered. With successive injections of larger doses, further acceleration of feather growth was obtained (Table II). After 2 administrations of 1.5 mg thyroxin with an interval of 6 days between injections, all areas attained or exceeded feather growth rates observed in normal hens. The posterior breast reached a higher growth rate than the other areas, though the period of acceleration was for a longer period in the saddle.

As a consequence of thyroidectomy in brown Leghorn hens there is a marked reduction in feather growth rates and an alteration in the order of growth-rate relations between the various body areas. A decrease in estrogen production[10] is probably responsible for the

[10] Blivaiss, B. B., and Domm, L. V., *Anat. Rec.*, 1942, **84** (Suppl.), 79.

[11] Wang, H., *Physiol. Zool.*, 1945, **18**, 335.

[12] Blivaiss, B. B., 1946, unpublished data.

assumption in such individuals of a growth-rate order of feather areas typical of gonadectomized males[6,11,12] and females.[12] The feather areas of athyroidic hens following thyroxin administration reveal the characteristic threshold differences.

15510

Relaxation of the Pubic Symphysis in Guinea Pigs Following Injections of Desoxycorticosterone Acetate.*

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The recent reports[1] on the concentration and purification of relaxin have led to a renewal of interest in the relaxation of the pelvis symphysis of guinea pigs. It seems progesterone acts on the uterus to release relaxin, which acts on the pelvis and relaxes the symphysis pubis. Since progesterone and desoxycorticosterone induce similar responses, such as prolongation of life and growth in adrenalectonized animals,[2-4] progestational changes in the uterus[5] and growth of the lobules of the mammary glands,[6] it seems likely that desoxycorticosterone could also induce relaxation of the pelvic symphysis of guinea pigs. This report contains data supporting this view.

Methods. Forty-eight virgin oophorectomized guinea pigs were used in this study. They were injected as outlined below, and after a recovery period of at least 2 weeks, they were sometimes used again for these experiments. The estrogens† in the form of theelin, and stilbestrol, were given in olive and peanut oils, and injected subcutaneously daily, for 2 days. The site of injection was on the shoulder and back, as this area[7] gives less oil cysts than any other area commonly used for injecting oils. Four days after the estrogens were started, progesterone and desoxycorticosterone acetate in olive oil were given, either intramuscularly or intraperitoneally.

Pelvic examination was gently carried out while the guinea pig rested quietly on one's lap or knee. The state of pelvic relaxation was determined by manual manipulation of the tuber ischi; these were held between the middle finger and thumb of each hand, while one of the fingers was pressed against the symphysis to determine the extent of movement present. By this method, there was almost no danger of mechanically forcing the pelvic ligaments to yield. The degree of movement was recorded from one to 4+, and those cases of 2+ or more, were considered to be positive for pelvic relaxation. Since the chief interest was to know whether the pubic symphysis was or was not relaxed, it seemed unnecessary to arrange the response into further gradations.

Fluoroscopic examination was also carried

* Research paper No. 597, Journal Series, University of Arkansas.

1 Hisaw, F. L., Zarrow, M. X., Money, W. L., Talmage, R. V. N., and Abramowitz, A. A., *Endocrinology*, 1944, **34**, 122.

2 Emery, F. E., and Schwabe, E. L., *Endocrinology*, 1936, **20**, 550.

3 Gaunt, R., and Hays, H. W., *Am. J. Physiol.*, 1938, **124**, 767.

4 Schwabe, E. L., and Emery, F. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 383.

5 Van Heuverswyn, J., Collins, V. J., Williams, W. L., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 552.

6 Mixner, J. P., and Turner, C. W., *Endocrinology*, 1942, **30**, 706.

† Appreciation for supplies is recognized as follows: Theelin, Dr. Oliver Kamm, Parke, Davis and Company; progesterone and desoxycorticosterone acetate, Dr. Erwin Schwenk, Schering Corporation.

7 Emery, F. E., Matthews, C. S., and Schwabe, E. L., *J. Lab. and Clin. Med.*, 1942, **27**, 622.

TABLE I.

No. of tests	Estrogens, mg	Progesterone, mg	% positive	No. of tests	Estrogens, mg	Desoxycorticosterone acetate, mg	% positive
10	2 th	1½	70	10	2 th	1½	50
5	5 th	1½	75	5	5 th	1½	75
7	2 S	1½	29	7	2 S	1½	43
10	5 S	1½	40	10	5 S	1½	50
5	5 S	5	100	5	5 S	5	75
			Summary				
37			58	37			57

Under estrogens in Column 2, th—theelin, S—stilbestrol. The per cent of guinea pigs showing a positive relaxation of the pubic symphysis is shown in the last column. Body weight range—300 to 580 g.

out, and this helped to confirm the amount of separation that occurred between the bones at the pubic symphysis.

Results. The dosages of estrogens employed were probably above the threshold necessary to prepare the uterus and pubic symphysis for the action of progesterone and desoxycorticosterone acetate. This is shown in Table I by the fact that the per cent of guinea pigs showing relaxation of the symphysis pubis, induced by 1.5 mg of either progesterone or desoxycorticosterone acetate, was about the same in animals primed with either 2 or 5 mg of estrogens.

It will also be noted in Table I that there is no noticeable difference between progesterone and desoxycorticosterone in bringing about relaxation of the pubic ligaments of guinea pigs.

When the total dose of progesterone, or desoxycorticosterone is 5 mg, the relaxation response is obtained in almost all animals, but the degree of relaxation was not always maximal. Likewise, much smaller doses sometimes produced what may be called a maximal response. The degree of relaxation of the pubic symphysis in some of these guinea pigs was equal to that found in advanced pregnancy, as shown by both manual and fluoroscopic examinations of the pelvis.

Both progesterone and desoxycorticosterone induced changes in the mammary glands, sufficient for lactation, and in some cases, large milk drops were pressed from them. Sometimes this was observed as early as 24 hours following the injection, but it usually occurred later, and as long as 4 days in one case. Although these time intervals also fit the time necessary for relaxation of the pubic symphysis, the 2 phenomena seem unrelated and occur independently of each other.

Discussion. Previously[8] it was found that progesterone could substitute for desoxycorticosterone acetate on an equal weight basis when tested on the growth curve of adrenalectomized rats. But as previously discussed, various investigations have found the dose of progesterone necessary to maintain life in immature adrenalectomized rats, varied from 1 to 4 mg.

In fact in the same volume of *Endocrinology* as the above citation, it was shown[9] that in cats the progestational action of progesterone was about 10 times that of desoxycorticosterone acetate. Changes in blood, sugar, and serum electrolytes also require larger doses than needed to maintain life in adrenalectomized animals.[10,11] In the present study it was sometimes found that in identically treated guinea pigs some were well relaxed while others gave no response. Thus, it is apparent that on a weight basis these 2 compounds vary greatly in their different actions, and therefore, a conservative view of the data, (Table I), seems to be that desoxycorticosterone acetate can substitute for progesterone in the reaction involved in the loosening of the pelvic symphysis of the guinea pig, but that the exact dosages have yet to be determined.

[8] Emery, F. E., and Greco, P. A., *Endocrinology*, 1940, **27**, 473.

[9] Leathem, J. H., and Crafts, R. C., *Endocrinology*, 1940, **27**, 283.

[10] Harrison, H. E., and Harrison, H. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 506.

[11] Thorn, G. W., Howard, R. P., and Emerson, K., Jr., *J. Clin. Invest.*, 1939, **18**, 449.

The palpatory method for determining the extent of relaxation of the pelvic symphysis is not very precise, although in experienced hands, the movement in the pelvic symphysis is readily felt. Fluoroscopic examination helps in determining a positive response, but it is doubtful if this method is as reliable as palpation. The X-ray has given additional data and, as judged from a recent report,[12] it is quite reliable in detecting changes in the pelvis of mice.

Summary. In oophorectomized guinea pigs primed with estrogens, desoxycorticosterone acetate can substitute for progesterone in bringing about relaxation of the pubic symphysis. Only a few animals showed a degree of relaxation comparable to that obtained in advanced pregnancy. Milk secretion was obtained in many of these guinea pigs.

[12] Hall, K., and Newton, W. H., *J. Physiol.*, 1946, **104**, 346.

15511

Roentgenographic Studies of the Normal Human Gallbladder.

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This preliminary study was made to ascertain how the diminution in size of the gallbladder shadow is accomplished following a fatty meal.

Method. Twelve healthy individuals were chosen, 6 males and 6 females, aged 20 to 27 years. Following a fat-free evening meal, 3 g of Priodax (beta-(4-hydroxy-3,5-diiodophenyl)-alpha-phenyl-propionic acid) was given orally.[1] Only fruit juice, tea, black coffee, and water were allowed until 14 hours later. Then after a cleansing enema the first film (A) was taken. Following this a meal consisting of 8 ounces of cream with yolks of 2 eggs was given. A second film (B) was taken one hour later, and a third (C) 4 hours after the first. All exposures were made in the upright position, in neutral respiratory phase, after the gallbladder had been located by fluoroscopic examination.

Roentgenographic Data. In all instances the gallbladder was clearly visualized on the first films (A). The size of the gallbladder varied in different individuals. The shadow appeared to have a greater density at its most dependent, fundic, portion, decreasing in density toward the uppermost, collic, portion, which was usually indistinctly outlined (Fig. 1-4).

On the second films (B), taken one hour after ingestion of the fatty meal, the gallbladder shadow was invariably present, although in 2 instances it was faintly outlined. In 3 instances the decrease in size of the shadow was negligible. In the remaining 9 instances, the shadow was decreased in size about 10 to 50%. The density of the shadow was usually increased and was more uniform, with clearer outline of the neck (Fig. 1-4).

On the third films (C) taken 4 hours after the fatty meal and 3 hours after the second exposure, the shadow of the gallbladder was present in all instances except one. In 6 instances the shadows decreased in size about 50 to 75% as compared to the first films. In 5 instances there was only slight diminution in size of the shadow, and there was a distinct difference in the density between the fundic and collic portions of the gallbladder, the fundic portion being more dense and more sharply outlined (Fig. 3 and 4). In 3 of these the shadows closely resembled in size and shape those on the first films (Fig. 2 and 4).

Interpretation. The ingestion of the Priodax, according to present knowledge, is followed by the appearance of the radio-

[1] Einsel, I. H., and Einsel, T. H., *Am. J. Digest. Dis.*, 1943, **10**, 206.

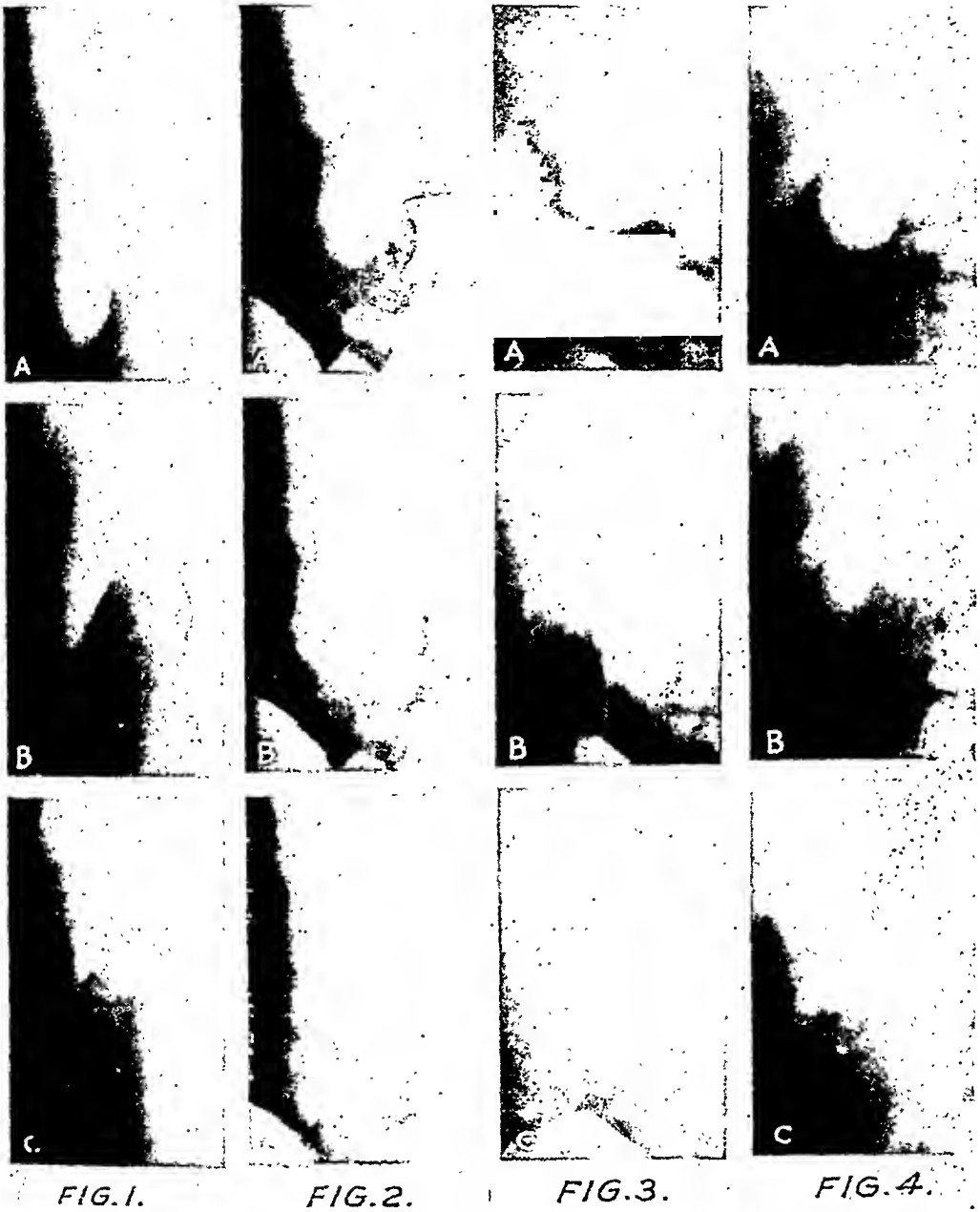

Fig. 1 to 4.

Films A were taken 14 hours after the ingestion of Priodax, B one hour later following the fatty meal, and C 4 hours after the first films. Case 1, 5, 7, and 9, respectively.

opaque substance in the bile. Information is not available as to how long the Priodax is present in the bile coming from the liver and in what concentrations. Experimental data on other substances, methylene blue,[2,3] tetraiodophenolphthalein, eosin, erythrosin and rose bengal,[4] provide information as to the rate of their excretion in the bile and their

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[12] Hall, K., and Newton, W. H., *J. Physiol.*, 1946, **104**, 346.

15511

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[1] Einsel, I. H., and Einsel, T. H., *Am. J. Digest. Dis.*, 1943, **10**, 206.

15512 P

Ovulation in Non-Lactating Puerpera.

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Menstruation ordinarily reappears in the nonlactating puerperal woman prior to ovulation. Long after the lochia has ceased, anovular bleeding appears, but usually is followed within a few weeks by ovular menstruation. Griffith and McBride,[1] on the basis of endometrial biopsy studies, reported that ovulation was delayed until 5 months or later postpartum in a group of 11 nonlactating patients. These same women showed anovular menstruation at an average of 2 months postpartum.

The present study was undertaken in order to determine the time of reappearance of ovulation in the nonlactating puerpera by means of the basal body temperature technic.[2] This method of evaluation agrees with direct evidence of ovulation observed at laparotomy.[3] Twenty-three nonlactating puerpera charted their basal temperatures until ovulation and menstruation recurred regularly. Endometrial biopsies were taken during the phase of temperature elevation in 7 of the group, and all of these showed progestational or secretory changes. Menstruation occurred within 2 weeks of the time of biopsy in each case.

As shown in Table I the average time of 8.4 weeks (range 5-15 weeks) for the reappearance of menstruation, agrees with Griffith and McBride's figure of 8 weeks. A significant difference will be noted, however, in the estimated average time of ovulation, which is less accurately determinable. Although the initial menstruation is almost always anovular, a few patients in this series ovulated prior to menstruation, and their data were. obviously, partially responsible for the low figure of 10.2 weeks (range 6-17 weeks) as contrasted with 20 weeks reported by Griffith and McBride for ovulation.

During the course of these studies, it was observed that when lactation was not carried on, or when it was discontinued very early, anovular bleeding was usually seen prior to the return of ovulation. However, after about the eighth postpartum week ovulation occurred with increasing frequency prior to menstruation. These observations will presumably find explanation in the availability of distinct proportions of pituitary gonadotrophins, adequate in some cases to stimulate the normal cyclic growth sequence in the ovary, culminating in ovulation, and deficient in other instances in which the follicular apparatus does not for a time mature and ovulate, but does form enough hormone to bring about cyclic uterine changes. The appearance of ovulation in these instances seems to imitate the succession of events found at the menarche in that the cycles progressively improve until they resemble the normal pattern. Ovulation probably requires more gonadotrophin than menstruation, and the variation in the amount available in different puerpera during the early weeks of the puerperium is relatively large.

Summary. From the study of basal temperatures. correlated with endometrial biopsy

TABLE I.
Reappearance of Ovulation and Menstruation in the Nonlactating Puerpera.

No. of cases	Onset menstruation in avg weeks post-partum	Onset ovulation in avg weeks post-partum
23 (this report)	8.4	10.2
11 (Griffith and McBride)	8.0	20.0

[1] Griffith, L. S., and McBride, W. P. L., *J. Mich. M. Soc.*, 1939, 38, 1064.

[2] Rubenstein, B. B., *Endocrinology*, 1938, 22, 41.

[3] Greulich, W., Morris, E. S., and Black, M. E., *Proc. Conf. Prob. Human Fertility*, 1943, p. 37.

accumulation in the gallbladder. According to experiments with one of these substances (methylene blue), the gallbladder can resorb half the volume of its fluid content per hour.[5,6] There is probably a close similarity between the behavior of these substances and that of Priodax. During the period in which radio-opaque substance is present in the bile, some of it reaches the gallbladder. There the opaque substance is resorbed more slowly than the bile and so it eventually attains sufficient concentration to cast a shadow on an X-ray film.

All films in this series were taken in the upright position. The shadow cast by the gallbladder content therefore more closely approximates the anatomic outline of the viscus than that in films taken in the horizontal position.

At the time the first films were taken, neither the hepatic, the common bile, nor the cystic ducts were visualized. Only the gallbladder contained the opaque substance in high enough concentration to cast a shadow. The same situation prevailed at the time of the second and also of the third films. Since in each instance the shadow on the second film changed its contour slightly and its size decreased 10 to 50% of the original and became more dense, it is safe to conclude that practically none of the opaque substance left the gallbladder between the time of the first and second exposures. The presence of sufficient opaque substance to visualize the viscus 4 hours after ingestion of the fatty meal would also support the contention that no loss by evacuation of the gallbladder content through the cystic duct necessarily occurred between the time of the second and third exposures.

Evacuation through the cystic duct of any considerable quantity of the gallbladder content would reduce the amount of radio-opaque substance remaining. Such a decrease of the opaque substance in the gallbladder, however, would cause the shadow not only to become smaller, but also more faint. Selective resorption, on the other hand, removes the solvent leaving the opaque substance in increased concentration, hence the increased density of the shadow on the second films.

A stimulation such as the fatty meal, which causes an increase of the flow of bile from the liver and into the duodenum, may temporarily decrease or increase the flow of bile into the gallbladder. In either event, the gallbladder continues to resorb half the volume of its content per hour. The change in contour, the reduction in size and the increase in density of the shadow as observed on the second films may, therefore, be explained by further concentration of the opaque substance while little or no new bile enters the gallbladder. The changes on the third films, 3 hours after the second, may be explained by the variations in the amount of new bile entering the gallbladder and diluting the opaque substance present. From the data so far obtained it may therefore be concluded that the diminution in size of the gallbladder shadow following the fatty meal is not necessarily caused, as currently believed, by evacuation of the gallbladder content but may be accomplished by selective resorption.

Summary. Cholecystographic studies on 12 healthy individuals were made in the upright position. The data so far obtained indicate that the diminution in size of the gallbladder shadow following the fatty meal may be accomplished not necessarily by evacuation but rather by selective resorption of the gallbladder content.

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[2] Halpert, B., and Hanke, M. T., *Am. J. Physiol.*, 1929, **88**, 351.

[3] Mills, D. R., and Halpert, B., *Am. J. Physiol.*, 1933, **103**, 265.

[4] Halpert, B., and Hanke, M. T., *Am. J. Physiol.*, 1932, **100**, 433.

[5] Halpert, B., Thompson, W. R., and Marting, F. L., *Am. J. Physiol.*, 1935, **111**, 31.

[6] Halpert, B., O'Connor, P. A., and Thompson, W. R., *Am. J. Physiol.*, 1935, **112**, 383.

TABLE I.
Effect of Nicotinine Acid and Tryptophane on Growth Retardation in Rats on Diets with Added Phenylalanine and Tyrosine.

Ration used	G gained per week* and range
Basal	19 (18-20)
" 1% *dl*-phenylalanine + 1% *l*(−)tyrosine	13 (8-19)
" 1% " + 1% " + 2 mg % nicotinic acid	11 (8-13)
" 1% " + 1% " + 100 " *l*(−)tryptophane	14 (11-18)
" 1% " + 1% " + 500 " nicotinic acid	16 (11-22)
" 1% " + 1% " + 100 " " " + 1% *l*(−)tryptophane	15 (13-17)
" 2% " + 2% "	1 (0.7-2)
" 2% " + 2% " + 100 mg % nicotinic acid	4 (3 -6)
" 2% " + 2% " + 1% *l*(−)tryptophane	4 (2.6-7)
" 2% " + 2% " + 500 mg % nicotinic acid	6 (4.6-7)
" 2% " + 2% " + 2% *l*(−)tryptophane	7 (5 -8)
" 1.5% *l*(−)tyrosine	12 (9-14)
" 4% "	2.4 (2- 3)
" 4% *dl*-phenylalanine	6 (4- 9)
" 100 mg % nicotinic acid	18 (16-19)
" 1% *l*(−)tryptophane	17 (16.6-17.6)

* Average of three weeks.

suggests that the effect produced by the addition of phenylalanine to a diet containing tyrosine may merely add to the effect of the tyrosine. Using deuterium-labeled phenylalanine, Moss and Schoenheimer[3] have shown that when a diet is supplemented with 2% phenylalanine, 20 to 30% of this is converted to tyrosine in the growing rat. The rate of conversion was not affected by the addition of an equal amount of tyrosine to the diet.

Lesions similar to those observed in the present study were previously described by Hueper and Martin[4] as a result of the addition of 10 parts of tyrosine to a diet containing 18 parts of casein and 100 mg % of nicotinic acid.

Corneal vascularization has been seen to occur in a variety of conditions including tryptophane deficiency,[5] lysine and methionine deficiency, and on diets low in protein.[6]

The growth retardation produced by the addition of 1% of phenylalanine and 1% tyrosine could not be counteracted by adding 2 mg % of nicotinic acid or 100 mg % of tryptophane to the diet (Table I). However, a very large amount of nicotinic acid (500 mg %) did appreciably decrease the growth retardation, as well as the appearance of external lesions. There seemed to be no advantage to the simultaneous addition of nicotinic acid and tryptophane.

The almost complete inhibition of growth by the addition of 2% phenylalanine and 2% tyrosine could be alleviated to some extent by the incorporation of 500 mg % of nicotinic acid or 2% of tryptophane to the diet. On the other hand, the addition of nicotinic acid or tryptophane to the basal diet had no growth stimulatory effects.

Two per cent phenylalanine and 2% tyrosine produced no deleterious effects when incorporated in the following diet: Argentine casein 25, Brewer's yeast 6, dried liver 4, sucrose 10, U.S.P. salt I 4, cooked starch 47.9, cellulose 3, and choline 0.1 parts per 100. Mixed tocopherols in corn oil were fed at a level of 11.6 mg per day.

The reason for the rather striking response of the rat toward a relatively low protein diet supplemented with phenylalanine and tyrosine is not known at the present time. It is hoped that experiments now in progress will aid in clarifying some unanswered questions. These experiments, however, serve to demonstrate possible complications arising

[3] Moss, A. R., and Schoenheimer, R., *J. Biol. Chem.*, 1940, **135**, 415.

[4] Hueper, W. C., and Martin, G. J., *Arch. Path.*, 1943, **35**, 685.

[5] Totter, J. R., and Day, P. L., *J. Nutrition*, 1942, **24**, 159.

[6] Sydenstricker, V. P., Hall, W. K., Hock, C. W., and Pund, E. R., *Science*, 1946, **103**, 194.

control, the average time for the initial ovulation in 23 nonlactating puerpera was found to be 10.2 weeks postpartum. There is considerable variation in the appearance time of ovulation in the absence of lactation. This may be attributed to varying degrees of gonadotrophic stimulation, sufficient in some cases to culminate in ovulation as early as the sixth postpartum week.

15513

Growth Retardation and Corneal Vascularization with Tyrosine and Phenylalanine in a Purified Diet.

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In a previous study dealing with the antagonistic action of certain amino acids toward the growth of *Streptococcus bovis* in a synthetic medium, it was found that a combination of *dl*-phenylalanine and *l*(-)tyrosine showed a marked inhibitory effect.[1] This inhibition of growth could be counteracted by the addition of *l*(-)tryptophane to the medium. Increased amounts of nicotinic acid added to the medium showed no effect. Krehl, Teply, Sarma, and Elvehjem[2] have shown that growth retardation in rats produced by the addition of corn grits to the diet could be counteracted by adding a small amount of tryptophane or nicotinic acid to the diet. In view of this fact the work with amino acid antagonisms was extended in order to determine whether the same phenomenon could be demonstrated in rats.

The following basal ration, similar to that used by Krehl *et al.*[2] was used: Labco vitamin-free casein 10, sucrose 83, corn oil 3, U.S.P. salt I 4, and cystine 0.15 parts. Vitamins were incorporated in the basal ration in the following amounts: thiamine 0.2, riboflavine 0.3, pyridoxine 0.25, calcium pantothenate 2.0, choline chloride 100, inositol 10, 2-methylnaphthaquinone 0.1, biotin 0.01, and folic acid 0.0115 mg per 100 g. Halibut liver oil (diluted 1:2 with corn oil) was fed at a level of 2 drops per week, with α-tocopherol included at 0.5 mg per drop. Additions to this diet were made by replacing an equal weight of sucrose. Substances added to the basal diet were thoroughly mixed with it using a mortar and pestle. Weanling, male rats averaging from 45 to 55 g were used throughout these experiments. Five rats were placed on each diet except in the case of a few control diets in which 3 were used.

The addition of 1% *dl*-phenylalanine and 1% *l*(-)tyrosine to the basal ration produced a substantial growth retardation (Table I) which was accompanied in about 50% of the cases by vascularization of the cornea and marked edematous swelling of the feet. With the addition of levels of phenylalanine and tyrosine as high as 2% each, growth was almost completely inhibited over a 3-week period. At this higher level more severe external lesions were noted and they occurred in 100% of the cases. Spontaneous healing of the external lesions was noted with all of the levels used in these experiments. When 2% phenylalanine and 2% tyrosine were used, the lesions began to appear on the 6th day and healing became apparent on the 15th to 18th day.

Contrary to the results obtained with *Streptococcus bovis*, it was found that the addition of tyrosine or phenylalanine alone to the basal ration produced growth retardation and external lesions to an extent which

1 Niven, C. F., Jr., and Washburn, Mary R., unpublished.

2 Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

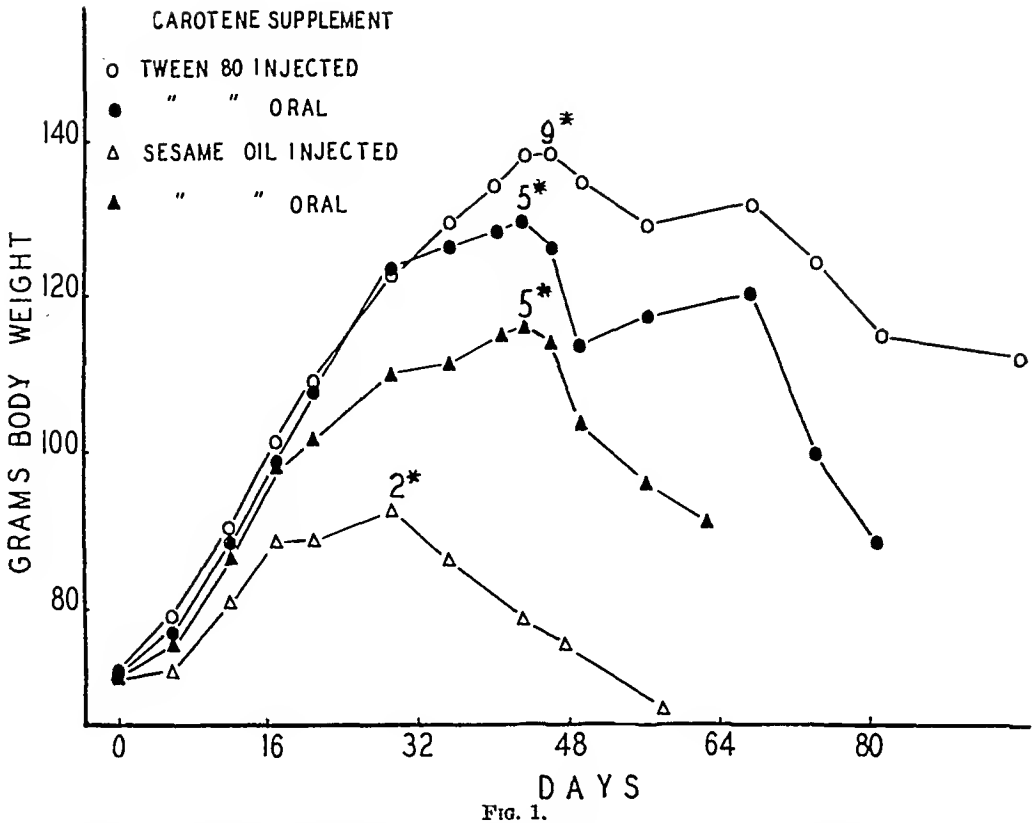

FIG. 1.

Growth of vitamin A depleted rats receiving single doses of carotene in sesame oil and in Tween 80, orally and intramuscularly.

(1) —○— 0.44 mg of carotene + 0.2 mg alpha-tocopherol in 0.1 ml Tween 80 intramuscularly. (9 rats; average survival time—71 days.)
(2) —●— Same as (1) but orally. (8 rats; average survival time—40 days.)
(3) —Δ— 0.44 mg of carotene + 0.2 mg alpha-tocopherol in 0.1 ml of Sesame oil intramuscularly. (8 rats: average survival time—20 days.)
(4) —▲— Same as (3) but orally. (9 rats; average survival time—40 days.)
* Number of rats alive at point of maximal growth.

The average survival time of the Tween-injected group exceeded that of either of the 2 orally supplemented groups; the time at which maximum growth was attained was about equal for the 3 groups. In Group 3, the carotene injected in oil was the most poorly utilized as judged by all criteria. After the death of each of the rats receiving an intramuscular injection, the thigh muscles were dissected and residual carotene was extracted and determined. The amount of carotene found remaining in the muscle tissue of the oil-injected rats varied from 27 to 76%, while only trace amounts were found in any of the Tween-injected rats.

TABLE I.

Recovery of 0.5 mg of Carotene in 0.1 ml of Tween 80 or Sesame Oil After a Single Injection into the Thigh Muscle of Rats.

Days after injection	% carotene* recovered from muscle	
	Tween 80	Sesame oil
2	7 (43)	98
7	trace (14)	63
15	none (20)	48
29	" (1)	33
37	"	18
65	"	21

* The number in the parentheses indicates the per cent of carotene recovered from subcutaneous tissue near the site of the injection. Only trace amounts were ever found in the subcutaneous tissue in the case of oil injections. Carotene was determined with the Beckman Spectrophotometer.

from diets containing certain amino acids in quantities which are out of proportion to that found in most natural proteins.

Summary. The addition of 1% *dl*-phenylalanine and 1% *l*(-)tyrosine to a purified diet containing 10% casein produced growth retardation and external lesions. Phenylalanine is converted to tyrosine in the animal and so may add to the effect of the tyrosine. The addition of relatively large amounts of nicotinic acid or *l*(-)tryptophane will appreciably alleviate the deleterious effects of these amino acids.

15514

Utilization of Intramuscularly Injected Carotene.

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Carotene administered parenterally has been shown to be poorly utilized.[1,2] Subcutaneous injection of carotene in oil solution[1] or intravenous, intraperitoneal or intrasplenic injections[2] of aqueous colloidal suspensions of carotene have failed to increase stores of vitamin A in the liver of rats. Deficiency symptoms and death have occurred while considerable amounts of the injected carotene were still present in the body.[1,2] Studies in this laboratory of intramuscularly-injected carotene in oil solution have yielded similar results. Carotene in sesame oil, with and without added tocopherols, when injected intramuscularly, has been found to restore growth to depleted rats for a short time only. Upon ensuing death, a considerable fraction of the carotene was found at the site of injection. After being solubilized in water by means of a suitable agent, intramuscularly-injected carotene was as efficiently utilized as carotene administered orally.

Experimental Methods and Results. When carotene is dissolved in Tween 80, (polyoxyalkylene derivative of sorbitan monoleate†) the solution may be diluted indefinitely with water without precipitation of the carotene. A comparative study of the absorption of carotene in Tween 80 solution and in sesame oil solution was made. 0.5 mg of carotene and 0.2 mg of α-tocopherol in 0.1 cc of solvent was injected into the thigh muscles of normal adult rats and the rate at which the carotene passed from the muscle determined. The data are presented in Table I.

The curative effect of intramuscularly-injected carotene as compared with that administered orally was measured by a study of the growth and duration of cure of vitamin A-depleted rats. Seventy male weanling rats were fed the U.S.P. XII vitamin A free diet. After 6 weeks on this diet, when cessation of growth and the appearance of ocular symptoms had marked the depletion of body stores of vitamin A, 40 of the most typically deficient rats were divided by weight into 4 groups. Rats dying within 3 days after receiving the supplement were not included in the data. From a comparison of the growth curves and the data on duration of cure, (Fig. 1) it may be seen that an intramuscular injection of carotene in sesame oil (Group 3) is poorly utilized as compared with the utilization of the same supplement given orally (Group 4). When the carotene was injected in Tween 80 (Group 1) the curative effect was as good as, if not better than, that obtained in the groups receiving carotene orally, either in Tween 80 or oil (Groups 2 and 4).

* The authors wish to acknowledge the technical assistance of the Misses M. Stoneman and F. Shitamae.

1 Lease, V. G., Lease, E. J., Steenbock, H., and Baumann, C. A., *J. Lab. and Clin. Med.*, 1942, **27**, 502.

2 Sexton, E. L., Mehl, J. W., and Deuel, H. J., *J. Nutrition*, 1946, **31**, 299.

† Atlas Powder Co.

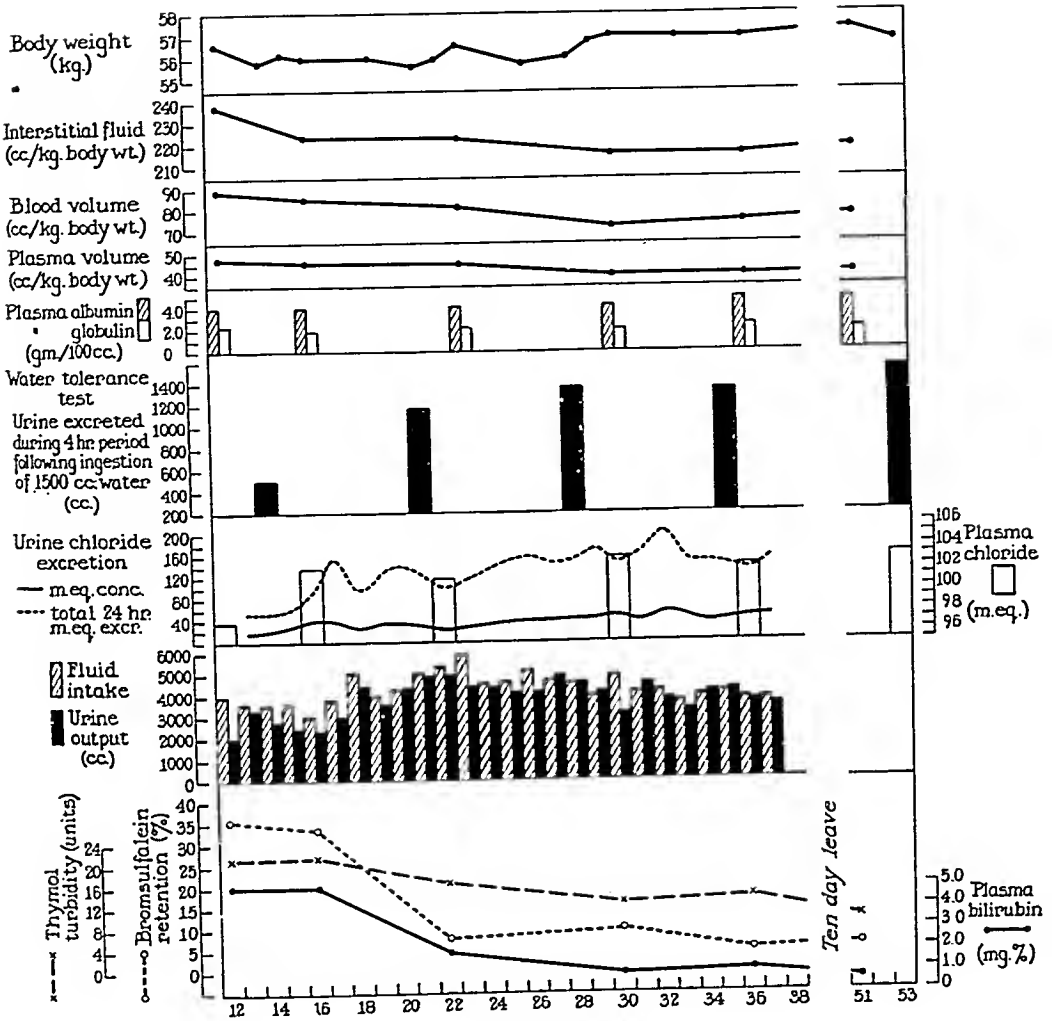

CHART 1.

with previous good health, whose disease course proceeded without complication. In each case, the diagnosis was obtained by a careful history and physical examination and confirmed by appropriate liver function tests. Throughout the study rigid water-balance conditions were maintained. This consisted of the daily determination of body weight and careful measurements of fluid intake and urine output. An attempt was made to keep the diet and salt intake constant for each patient. Measurements of plasma volume and interstitial fluid were determined at 4- to 7-day intervals, according to the simultaneous method of Gregerson and Stewart[6] in which the dye T1824 and sodium thiocyanate measured in the Coleman, Jr. spectrophotometer[7] were used. In this study the interstitial fluid volume was derived by subtracting the plasma volume from the total thiocyanate space. Although this fails to account for the small amount of red blood cell water, there was

[6] Gregerson, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

[7] Gibson, J. G., Jr., and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, **16**, 301.

Discussion and Summary. These experiments show definitely that carotene administered parenterally may be effectively utilized provided the carotene has been water-solubilized by solution in Tween 80. In such a solution not only is there a much more rapid transport of carotene from the site of an intramuscular injection, but there also occurs an effective conversion to vitamin A as demonstrated by the resumption of growth in vitamin A-depleted rats. These findings offer further support to the accumulating evidence[2,4] that the state of dispersion of the carotene is of importance for participation in the physiological processes of absorption, transport and enzymatic conversion to vitamin A.

3 Tomarelli, R. M., and György, P., *J. Biol. Chem.*, 1945, **161**, 367.

4 Greaves, J. D., and Schmidt, C. L. A., *Am. J. Physiol.*, 1935, **111**, 492.

15515

Alterations in Body Fluids During Acute Infectious Hepatitis.

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It has long been known that convalescence in acute liver disease may be initiated by a diuresis. Jones and Eaton[1] in noting this phenomenon in an unselected group of patients with acute and subacute liver disease suggested that it may have a favorable prognostic significance. To imply that recovery from liver disease is associated with the production of a large volume of urine, suggests that during the acute phase of the infection there may be profound disturbances in water metabolism involving increased water storage and possibly alterations in the transport and excretion mechanisms. Recognition of the intimate relationship existing between hepatic and renal physiology has impelled clinicians to speak of the ill-defined and little understood "hepato-renal syndrome." The suppression of urine volume in "catarrhal jaundice" and acute yellow atrophy are old observations,[2] as is the oliguria accompanying cirrhosis of the liver, especially when associated with ascites.[3] It is conceivable, therefore, that the latter may in part represent a more serious extension of the same mechanism to an advanced period in the development of chronic liver disease. Indeed, recent evidence suggests that the movement of body fluids during liver disease may be under endocrine control.[4] No available data exist that reveal the precise alterations of the fluid compartments of the body during the course of acute liver disease. Jones and Eaton[1] suggested that the observed diuresis of convalescence was a consequence of improved hepatic efficiency, resulting in a "shift of fluid from the tissues or serous cavities to the blood stream with the ultimate establishment of diuresis." Since adequate methods now exist for the simultaneous determination of approximate interstitial fluid (thiocyanate space), plasma volume and total blood volume, it was believed that a study of the distribution of body fluids during the course of acute infectious hepatitis would be of profit. In this disease one may work in a rapidly shifting metabolic scene.

Materials and Methods. Fourteen patients were studied as part of a group of acute infectious hepatitis cases observed in the Rockefeller Hospital.[5] All were young, adult males

* Dr. Hoagland died Aug. 2, 1946.

1 Jones, C. M., and Eaton, F. B., *New Eng. J. Med.*, 1935, **213**, 907.

2 Van Noorden, C., *Pathology*, 1907, **2**, 267.

3 Adler, A., *Klin. Woch.*, 1923, **2**, 1980.

4 Ralli, E. P., Robson, J. S., Clarke, D., and Hoagland, C. L., *J. Clin. Invest.*, 1945, **24**, 316.

5 Hoagland, C. L., and Shank, R. E., *J. A. M. A.*, 1946, **130**, 615.

chloride appeared in the urine, reaching a peak also on the 17th day, despite the fact that the daily calculated salt intake had been constant (9-10 g) since admission. This same period was further characterized by improvement in liver function. A slight but continued fall in the volume of interstitial fluid, total blood and plasma volumes was noted. On the 21st day of the disease a water tolerance test yielded more than double the previous amount of urine, 1170 cc being obtained. The period from the 21st to the 30th day of disease was characterized by an active diuresis and with this there followed stabilization of the interstitial fluid volume, plasma and blood volumes and a measurable improvement in liver function. Following this period the urine output fell slightly, but plasma chloride values and urine chloride excretion remained at sustained high levels. Little change was noted in the results of the water tolerance tests following the period of diuresis. With the restitution of liver function the initial fall in body weight was overcome and there now appeared a weight gain, despite the fact that the daily caloric intake had been maintained at values between 3000 to 3500 calories since admission. At the time of discharge from the hospital the patient had made a complete recovery, as indicated by maintenance of body weight and restoration of liver function. In addition there had been no significant change in the body fluids, which had been stabilized since the 30th day of the disease. Throughout the period of hospital observation, clinical signs of dehydration were never encountered and no peripheral edema or ascites had been noted.

Discussion. If the convalescent values are used as a base line, it is apparent from the data that a large interstitial fluid compartment exists during the acute phase of infectious hepatitis. In addition there is a tendency to retain or store ingested water, as reflected during this period by the poor urine output of the water tolerance test. That this does not represent inadequate renal function is supported by the fact that numerous routine examinations of the urine for albumin, sugar and formed elements in the sediment were always negative, though no formal renal function tests were performed. In the presence of an increased interstitial fluid compartment, as well as an increased plasma and blood volume, it is difficult to attribute the low urine volume, depressed urine chlorides and tendency to store ingested fluid to simple dehydration.[15] As suspected by Jones and Eaton, this increased interstitial compartment persists until improvement in liver function is manifest. With the mobilization of stored interstitial fluid there is a fall in body weight quite parallel to the amount of fluid lost. A great amount of chloride is apparently withdrawn and resides in this compartment in compliance with the laws of diffusion equilibria. This probably accounts for the low initial plasma chloride and depressed urine chloride excretion. This phenomenon has been previously noted in animals with acute liver injury produced experimentally by means of arsphenamine.[16] When mobilization of the interstitial compartment occurs there is an increase in both urine chloride concentration and total urine chloride excretion. With this adjustment the plasma chloride rises and following the diuresis that may appear at this time, there is less tendency to store ingested water as measured by the water tolerance test. The events of this period are probably set in motion by an improvement in hepatic efficiency. With the establishment of convalescence the body fluid compartments move toward stabilization. There follows a rise in plasma protein values somewhat greater than can be accounted for by the slight fall in plasma volume; this probably reflects improved protein synthesis by the liver. The changes in plasma proteins that may occur independent of alterations in plasma volume have been reported experimentally and are to be found especially where nutritional features are of great importance.[17]

The forces underlying the dynamics of

[15] Lyons, R. H., Jacobson, S. D., and Avery, N. L., *Am. Heart J.*, 1944, **27**, 353.

[16] Soffer, L. J., Dantes, A. D., and Sobotka, H., *Arch. Int. Med.*, 1937, **60**, 509.

[17] Mellors, R. C., Muntwyler, E., Mautz, F. R., and Abbott, W. E., *J. Biol. Chem.*, 1942, **144**, 785.

too little change throughout the course of the disease in the red blood cell volume, as measured by the venous hematocrit, to be of significance.[6] Total blood volume was calculated from the plasma volume by means of the venous hematocrit taken on the same sample.[8] The plasma bilirubin was determined according to the method of Malloy and Evelyn adapted to the Coleman, Jr. spectrophotometer.[9] This, together with the bromsulfalein test,[10] measurements of the plasma protein fractions and the thymol turbidity reaction of the serum,[11] were the indices of choice in following the state of liver function at frequent intervals. The total plasma protein and albumin and globulin fractions were determined by the micro-digestion method, in which Nesslerization[12] was used. Normal values for this method are: total protein, 6.0-8.0 g %; albumin, 3.5-5.0 g %; globulin, 1.5-3.0 g %; A/G ratio, 1.5-3.3. Blood specimens for plasma chlorides were collected under oil and measured according to the method of Wilson and Ball.[13] To disclose any tendency to store fluid, a water tolerance test was utilized at weekly intervals.[14] This is a simple expression of the volume of urine excreted under basal conditions in the 4 hours following the rapid ingestion of 1500 cc of water. This test was not performed on the days that the total volume of blood, plasma and interstitial fluid were measured. Preliminary data in the 14 cases are sufficiently constant so that the detailed presentation of one typical case will serve as a text adequate to illustrate the general quality and direction of movement of the body fluid pattern observed in the entire group.

Results. Chart I represents the serial data obtained from a typical case. The patient was a 34-year-old male, seen on the 12th day of his disease. The prehospital period had been marked by malaise, generalized muscular aches and pains, slight anorexia and nausea. There was one mild attack of vomiting 5 days before admission, but the patient had been able to take adequate food and fluids since that episode. Dark urine had appeared 7 days before and visible icterus 3 days prior to hospital admission. Physical examination revealed conspicuous icterus, and an enlarged tender liver. The patient's hospital course was afebrile and with the prompt institution of bed rest and adequate nutrition, convalescence proceeded without unusual event. Measurements of body weight and fluid balance, as well as determinations of blood and plasma volume and interstitial fluid volume were begun the day following admission. It may be noted from the accompanying chart that during the first 2 hospital days there was a fall of 0.8 kg in body weight. This period and the 2 days that followed were marked by the greatest fall in interstitial fluid volume from 237 cc/kg to 223 cc/kg of body weight. The value of 215 cc/kg attained later during convalescence may be taken as a base line. There were slight changes in the same direction but of less magnitude in the blood and plasma volume. Measurements of liver function revealed great impairment as indicated by greatly elevated values for the total biliruhin. the bromsulfalein test and an abnormal thymol turbidity reaction of the serum. It was impressive that despite an adequate fluid intake the urine volume was small compared to the urine output of later convalescence. At this time also the total plasma chloride as well as the urine chloride concentration and total daily urine chloride excretion were low. A water tolerance test done during the first week of admission yielded only 503 cc of urine after the ingestion of 1500 cc of water. By the 17th day of the disease there developed such a marked increase in urine volume that the patient was eliminating between 85 and 90% of his fluid intake. Commensurate with this, greater amounts of

8 Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, **16**, 547.

9 Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.*, 1937, **119**, 481.

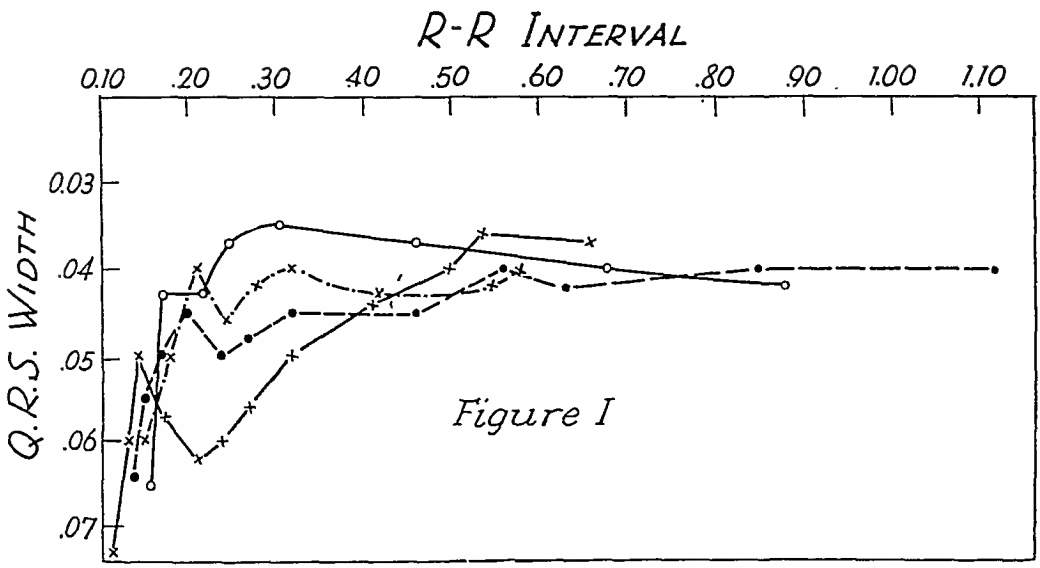
10 Mateer, J. G., Baltz, J. I., Marion, D. F., and MacMillan, J. M., *J. A. M. A.*, 1943, **121**, 723.

11 Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, **162**, 133.

12 Archibald, R. M., personal communication.

13 Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, 1928, **79**, 221.

14 Aldersberg, D., and Fox, C. L., *Ann. Int. Med.*, 1943, **19**, 642.



Four recovery curves of intraventricular conduction in the isolated rabbit heart.

variation in the rate. The overflow of the perfusate ran into a beaker, kept at a constant level by suction, and was returned to the reservoir. Electrodes for the electrocardiographic records were placed in this beaker. Stimuli were delivered to the ventricle at a rate first approximating the idioventricular rate; they were increased by stages up to about 400 per minute, or until some were blocked, or until transient paroxysmal ventricular tachycardia developed.

The QRS width was measured with the Cambridge Record Measuring Instrument. This was plotted against the time interval between stimuli, which was taken as an index of recovery time. Satisfactory preparations beat well for several hours; the experimental data were usually obtained within 60 minutes. Effects of fatigue were further excluded by repetition of both control and drug measurements at various intervals.

Results. Effect of varying rates. Several typical examples of the curves obtained are shown in Fig. 1. Some recovery curves show, in the range of slow rates, gradual shortening of the QRS with increasing rates; more common, however, is gradual prolongation with more rapid rates. A consistent observation is improvement in intraventricular conduction over a short range of rapid rates, causing a notch in the descending limb of the curve where the RR intervals are shortest. Immediately following the notch, however, with increasing rates, QRS widening becomes most marked.

A transient increase in aberration was usually noted in the second, third, and sometimes the fourth complexes of groups produced by sudden increases in rate; rapid shortening of relative refractoriness, as the rapid rate persisted, led to decrease in QRS width and distortion, with the maintenance of a constant QRS form and time for that rate. Similar findings have been made by Scherf[4] in experimental A-V block, and may be seen in patients who have paroxysmal tachycardia with partial A-V block.[5]

Effect of Strophanthin. Strophanthin-K (Strophosid, Sandoz)* was injected into the rubber connection of the cannula in a dose averaging 0.006 mg/kilo of weight of the intact rabbit. This caused a prolongation of the intraventricular conduction time (Fig. 2), which was greater with larger doses of the drug. This effect disappeared in approximately 15-30 minutes, with a return to the control values.

Effect of Quinidine. Quinidine sulphate was injected in a dose of 0.15 mg/kilo. Pro-

[4] Scherf, D., *Wien. Arch. inn. Med.*, 1929, **18**, 403.

[5] Decherd, G. M., Herrmann, G. R., and Schwab, E. H., *Am. Heart J.*, 1943, **26**, 446.

* These studies were supported, in part, by a grant from the Sandoz Chemical Works, Inc.

this changing fluid pattern may be suggested in outline. That there may be endocrine factors at work, such as an antidiuretic substance not effectively inactivated by a poorly functioning liver has been indicated.[4] The role of the pituitary in this mechanism has been suggested,[18,19] and requires further elucidation. Recent data, however, disclose a significant elevation in the level of excretion of biologically active estrogen in the urine during acute infectious hepatitis which attains normal values during late convalescence.[20] It is conceivable that movements in body fluid may follow these alterations in active estrogen and be mediated through a central agency such as the pituitary.

Summary. Interstitial fluid volume (thiocyanate space), total blood volume and plasma volume were measured at frequent intervals in 14 young adult males with acute infectious hepatitis. In each patient, the body fluid movement pattern was similar. Detailed data presented in one typical case disclose an increase in the interstitial fluid compartment during the preconvalescent period and a tendency to store ingested water as measured by a water tolerance test. Diminished plasma and urinary chloride values are also found during this period, probably because of chloride storage in the increased interstitial fluid compartment. With convalescence and the restitution of liver function, the interstitial fluid and its retained chloride are mobilized and the interstitial fluid volume falls to lower values. There is an accompanying fall in body weight and frequently a diuresis follows. Less tendency to store ingested fluid is also observed at this time. Plasma chlorides are now restored and urinary chlorides appear in normal amounts. A slight drop in plasma volume and total blood volume accompany these changes and appear to be independent of alterations in the plasma proteins.

It is suggested that these movements in the body fluids may have an endocrine basis.

18 Gilman, A., and Goodman, L., *J. Physiol.*, 1937, **90**, 113.

19 Ingram, W. R., Ladd, L., and Benbow, J. T., *Am. J. Physiol.*, 1939, **127**, 544.

20 Gilder, H., and Hoagland, C. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 62.

15516

Recovery Curves of Intraventricular Conduction; QRS Aberration.

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Aberrant intraventricular conduction, with change in form and duration of the QRS complex, is commonly encountered in paroxysmal tachycardia of supraventricular origin, and in premature beats from similarly located foci. Such aberration is regarded as being due to fatigue, or to inadequate recovery, of the conducting tissues. The recovery curves of A-V conduction have been carefully studied;[1] similar studies have been made of ventricular muscle strips, but no analogous data are available for the intact mammalian ventricle.

Method. We have used the isolated rabbit heart, perfused through its coronary vessels by a cannula tied into the aorta. The perfusion fluid was prepared according to the formula of Krebs and Henseleit;[2] it was kept at 37°C, and through it was bubbled oxygen containing 5% CO_2. The atria were cut off, and stimulating electrodes were pushed through the upper part of the I-V septum. The electrodes were activated by an electronic stimulator devised, and made for us, by Dr. S. A. Peoples.[3] This device permits variation in the strength of the stimulus, as well as wide

1 Decherd, G. M., and Ruskin, A., *Brit. Heart J.*, 1946, **8**, 6.

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3 Peoples, S. A., in press.

15517

Electrical Systole (Q-T Interval) of Rabbit Heart.

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The QT interval of the electrocardiogram encompasses the whole of activation and recovery of the ventricular muscle. Its duration varies with the heart rate. Logarithmic curves relating the QT interval to the preceding cycle length have been drawn by numerous authors;[1,2] a recent paper[3] attempts to establish a linear QT-RR relationship. The QT interval is prolonged in many varieties of heart disease, by myocardial anoxemia, by diminution in the blood calcium level, and by quinidine. Administration of digitalis shortens the QT interval, particularly when it has been prolonged by myocardial disease. Robb[4] has recently suggested that extra-cardiac factors may reflexly influence the QT duration.

In human patients, or in intact animals, the feasible variations in heart rate are limited. Hence we have studied the QT-RR relationship in the isolated perfused rabbit ventricle, using the technic previously described,[5] which permits variation in rate from that of the idioventricular pacemaker, 50-100/min., up to 400/min. or more. Strophanthin was injected into the perfusion system in doses approximating 0.006 mg/kilo of intact rabbit; then quinidine was employed in a dose of 0.15 mg/kilo. The effect of these drugs on electrical systole was studied.

Results. The figures show the forms of QT-RR curves obtained from this preparation. The QT interval is often prolonged by changes from very slow to moderate rates; further increasing rates uniformly shorten the QT measurement. The curve often flattens out at extremely rapid ventricular rates.

Quinidine, as has often been shown, always prolongs the QT interval markedly (Fig. 1).

The effect of strophanthin-K (Strophosid, Sandoz) was variable. When used in the above dosage, there was in some preparations prolongation of the QT interval; just as often shortening was obtained; several showed no appreciable change from the control curves. It was then found that when small amounts of strophanthin prolonged the QT interval, larger doses shortened it (Fig. 2).*

Comment. The QT-RR curves obviously are not linear, although in the central portion, excluding the slowest and fastest rates, the deviation from linearity may not be great. We have not attempted to derive formulæ that might fit the curves obtained.

If the QT interval measures, in its first part, intraventricular conduction, but in its greater portion, ventricular refractoriness, it is readily understood why increasing rates should shorten the QT duration, up to a limit at which further shortening becomes impossible. It is difficult, however, to explain on a similar basis the occasional slight prolongation of QT at moderate, as compared to very slow rates.

Clark and Mines[6] noted that strophanthin first prolonged, later shortened, the duration of the electrical response of the isolated frog heart. In human subjects, only the shortening has been observed to follow therapeutic dosage of digitalis glucosides. It appears that prolongation of the QT interval has been demonstrated only in isolated hearts; whether or not it may be found early in the course of gradual digitalization of humans has not yet been

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[2] Ashman, R., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 150.

[3] Schlamowitz, I., *Am. Heart J.*, 1946, **31**, 329.

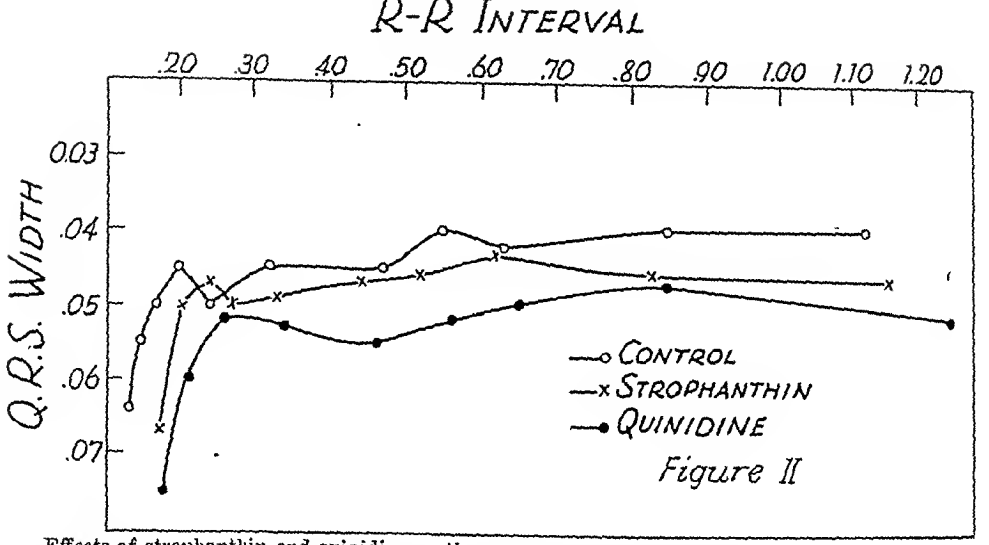
[4] Robb, J. S., *Fed. Proc.*, 1946, **5**, S7.

[5] Decherd, G. M., and Ruskin, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 114.

* Since the submission of these papers, we have been able to demonstrate similar effects of small and large doses of Cedilanid (Sandoz) and Seillaren-B (Sandoz), as well as digitoxin.

[6] Clark, A. J., and Mines, G. R., *J. Physiol.*, 1913, **47**, vii.

[7] Kisch, B., *Strophanthin*, Brooklyn Medical Press, 1944.



Effects of strophanthin and quinidine on the recovery curve of intraventricular conduction.

longation of conduction times, and of refractoriness, was uniformly produced.

Comment. With shortened periods of recovery the intraventricular conduction time is prolonged. This parallels the well-established recovery curve for A-V conduction. There is, however, one exception; at rapid rates there is a narrow range within which I-V conduction is temporarily improved. This phenomenon has not been noted in recovery curves of A-V conduction, unless it is analogous to the period of supernormal conduction sometimes found at the end of the period of relative refractoriness. Another possible explanation lies in the manner in which these values were determined. Recovery curves of A-V conduction are usually drawn from PR intervals of single complexes of varying degrees of prematurity, with a constant basic rhythm. Our own unpublished data, as well as Lewis and Master's,[6] show shortening of refractoriness and improvement of conduction with increasing rates. We have not measured, for the curves shown in the figures, the first few more aberrant complexes following rapid stimulation, but have utilized measurements made in complexes which represented a steady state of relative refractoriness at a given rate. The present data thus correspond to those which might be obtained from paroxysmal supraventricular tachycardia, rather than from single premature beats.

Aberrant wide QRS complexes are often seen after quinidine administration; the effect of digitalis is variable,[7] but aberration may occur. The effect of these drugs on conduction in the ventricle is, therefore, similar to their effect on the A-V conduction tissues. This impairment in conduction is probably effective throughout the myocardium, and cannot be assumed to pertain to clinical or experimental bundle branch block, where there is thought to be a localized region of defective conduction.

Rapid rates of stimulation of the ventricle, usually in the neighborhood of 350-400 per minute, fairly constant in the same, but varying in different hearts, often led to the persistence of ventricular tachycardia after cessation of the stimuli. The duration varied from a few seconds to a few minutes. In this preparation, and under the conditions thus far employed, we have been unable to note any effect of strophanthin in facilitating, or of quinidine in preventing or terminating, these paroxysms.

Summary. Data have been obtained from the isolated rabbit ventricle, from which have been drawn recovery curves of intraventricular conduction. Similar curves have been drawn after the injection of strophanthin and of quinidine.

[6] Lewis, T., and Master, A. M., *Heart*, 1925, 12, 209.

[7] Berliner, K., *Am. Heart J.*, 1931, 7, 189.

TABLE I.

Dose in mg HS	Liver P^{32} as % injected dose	% cells in mitosis
0.1	6.34 6.02 5.91	
	6.09	0.7
0.2	5.84 5.72 6.26	
	5.94	0.4
0.4	6.21 5.30 6.56	
	6.02	0.2
0.20 cc (propylene glycol only)	6.55 6.84 6.05	
	6.48	1.1

venously in doses not exceeding 1 mg Na_2HPO_4 and 1.5 microcuries per gram body weight. Three hours after the P^{32} injection, the livers were perfused with saline and removed. A small portion of the liver was used for P^{32} assay and the remainder for making a suspension of nuclei to obtain counts of the frequency of nuclei in mitosis. The nuclei were isolated as previously described[1] and the counts made in a counting chamber with Neubauer ruling. A minimum of 2,000 cells was counted for each value given in the tables. The results are given in Table I.

The differences in P^{32} uptake in HS-treated and in control animals are not significant. However, mitosis is markedly depressed and the inhibition of mitosis increases with increasing doses of HS. A dose of 0.1 mg of HS will reduce the mitotic rate to about one-half that of the control, while 0.4 mg reduces the rate to one-fifth of the control. Serial sections were made of the livers which on microscopic examination showed no pathological changes. The depression of the mitotic rate cannot therefore be attributed to a direct necrotizing action of the HS.

In a second series the 0.2 mg HS in propylene glycol was given at 4, 12, 16, and 22 hours after partial hepatectomy. All animals received P^{32} intravenously 24 hours after the partial hepatectomy and the livers were perfused and removed 3 hours later. There were 3-4 animals in each group. The results are summarized in Table II.

When the HS is injected 4 hours after partial hepatectomy, at a time before the cells have been stimulated to active mitosis (Group 1) the mitotic rate remains at the level observed in non-regenerating livers.[2] If the HS is given 22 hours after hepatectomy and allowed to act for 5 hours (Group 5) the mitotic rate is much reduced but not to a level as low as that of Group 1. The data may therefore be taken to indicate that if the HS gets to the cells before the conditions ultimately leading to mitosis are reached, it prevents the establishment of these conditions. The higher mitotic rates of Groups 3 and 4 as compared with Group 1 suggest either local destruction of HS and/or its reaction products or the removal of these substances from the site of action.

The values for percent P^{32} uptake are given with maximum deviations of individual values from the mean of each group. The mean in Group 4 is depressed by one unusually low value which is probably due to an experi-

TABLE II.

Group No.	Hrs after hepatectomy when solution was injected	Hrs with HS	% cells in mitosis	P^{32} %
1	4	23	0.1	3.9 ± 0.2
2	12 (propylene glycol only)	15	0.5	4.2 ± 0.5
3	12	15	0.3	4.2 ± 0.1
4	16	11	0.3	3.1 ± 1.7
5	22	5	0.2	5.0 ± 0.15

[1] Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 275.

[2] Marshak, A., and Byron, R. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 200.

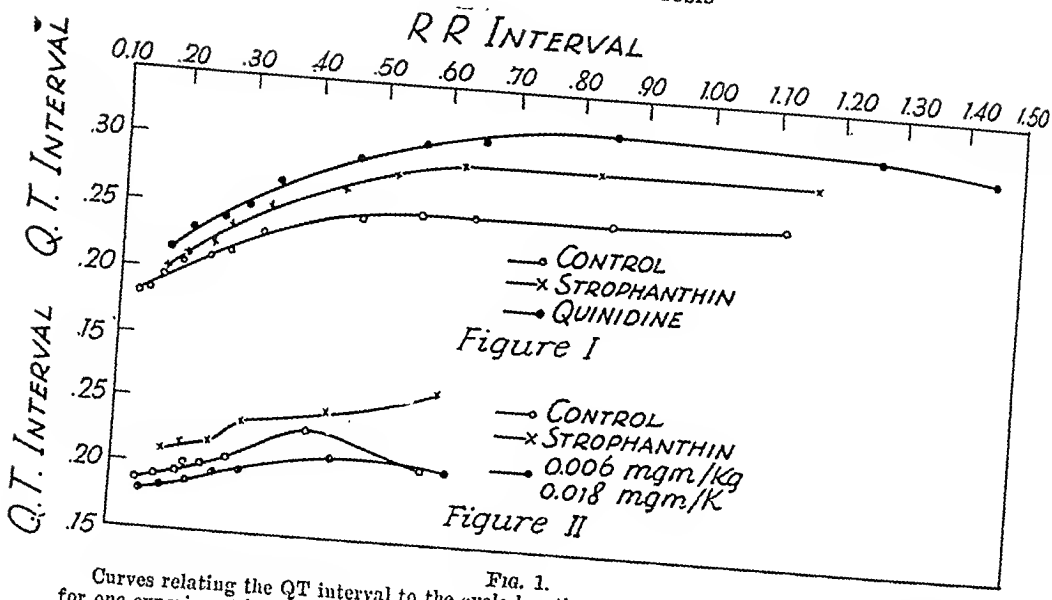


FIG. 1.

Curves relating the QT interval to the cycle length. The effect of digitalis and of quinidine for one experiment is shown.

FIG. 2.

QT-RR curves for a small, and for a large, dose of strophanthin.

determined. Strophanthin has been found,[7] like digitalis, to shorten the QT interval in animal experiments and in human patients. Although the action of quinidine in prolonging refractoriness, *i. e.*, the QT interval, parallels its effect in delaying intraventricular conduction as measured by the width of the QRS complex,[5] this parallelism is lacking for strophanthin, at least in the larger doses.

At fast rates strophanthin and quinidine produce relatively little deviation from the control curve; the effects of increased rates apparently minimize the influence of the drug. Conversely, the most marked changes in the QT interval, produced by these drugs, are usually noted at relatively slower rates of stimulation.

Summary. Curves have been drawn relating the QT interval to the cycle length for the isolated perfused rabbit ventricle. The effect of strophanthin and of quinidine on these curves has been studied.

15518

Effect of Mustard Gas on Mitosis and P^{32} Uptake in Regenerating Liver.*

A. MARSHAK.

From the Tuberculosis Control Division, U. S. Public Health Service.

Rats (1 month, 40-50 g) were partially hepatectomized and 24 hours later β*is* (β-chloroethyl) sulfide ("HS") was injected into the femoral vein. The HS was dissolved in propylene glycol, at a concentration of 2 mg per cc. (A dose of 2 mg per kilo killed rats in 3-4 days.) Ten minutes later P^{32} as Na_2HPO_4 was administered intra-

* The work described in this paper was done at the University of California under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California. It was undertaken at the request of the Chairman of Section 9:5 of the National Defense Research Committee.

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venously in doses not exceeding 1 mg Na_2HPO_4 and 1.5 microcuries per gram body weight. Three hours after the P^{32} injection, the livers were perfused with saline and removed. A small portion of the liver was used for P^{32} assay and the remainder for making a suspension of nuclei to obtain counts of the frequency of nuclei in mitosis. The nuclei were isolated as previously described[1] and the counts made in a counting chamber with Neubauer ruling. A minimum of 2,000 cells was counted for each value given in the tables. The results are given in Table I.

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[1] Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 275.

[2] Marshak, A., and Byron, R. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 200.

TABLE III.

	10 hrs HS	24 hrs HS	24 hrs cont.	48 hr HS	48 hr cont.
% mitosis	.19	.39	.61	.43	.33

mental error. Group 5 shows a somewhat higher P^{32} content but this is probably not significantly different from Groups 1 and 2.

In a third series, 0.2 mg HS was injected 24 hours after hepatectomy and allowed to act for 10, 24, and 48 hours. Mitotic counts were taken but P^{32} was not followed. Results were as shown in Table III.

Although mitosis is lower in livers examined 24 hours after HS administration than in controls, yet it is higher in those removed 48 hours after HS treatment. The results, as in the previous experiment, suggest that the HS inhibition is temporary, and, upon elimination of HS from the liver, the rate of mitosis increases. Since HS has no effect on the P^{32} uptake of the tissue comparable to that on mitosis, the results suggest that the inhibition observed is not produced by way of the cellular phosphorylating mechanism.

The inhibition of mitosis by HS suggested analogy with similar effects of x-rays which also inhibit mitosis in the nuclear resting stage. A search was therefore made for chromosome aberrations comparable to those found after x-ray treatment. None were observed. In an earlier study, it was shown that treatment with x-rays which also inhibits mitosis in the resting stage produces little if any change in the P^{32} taken up by liver or tumor tissue; however, the distribution of P^{32} between nucleus and cytoplasm was very markedly altered.[4] Obviously, therefore, further work is needed before final conclusions are reached on the effect of HS on the cellular and nuclear metabolism of phosphorus.

Summary. 1. Within 3 hours after intravenous injection, 0.1-0.4 mg of βis (β-chloroethyl) sulfide per 50 g rat produces a marked reduction in the number of cells in metaphase and anaphase in regenerating rat liver. A dose of 0.2 mg inhibits mitosis for at least 24 hours. By 48 hours, this inhibition is released. If the mustard reaches the liver before mitosis is initiated the cells are prevented from entering into mitosis.

2. There is no significant change in P^{32} uptake by liver cells during the interval 3-23 hours after the mustard is injected.

3. Comparison is made with the effects of X-rays on mitosis and P^{32} uptake. No chromosome abnormalities were observed at the dosage levels used.

[4] Marshak, A., *J. Gen. Physiol.*, 1941, **25**, 275.

15519

Application of Gersh's Ferrocyanide Technique to the Study of Experimental Renal Disease.

HAROLD E. HARRISON* AND HENRY BUNTING. (Introduced by M. C. Winternitz.)

From the Department of Pathology, Yale University School of Medicine.†

Gersh, using histochemical methods, demonstrated in the rabbit, dog, monkey, and other species[1,3] that sodium ferrocyanide was excreted solely by glomerular filtration without apparent tubular reabsorption or secretion;

* Now at the Department of Pediatrics, Baltimore City Hospitals, Baltimore, Maryland.

† The work described in this paper was done with the aid of a grant under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Yale University.

[1] Gersh, I., and Stieglitz, E. J., *Anat. Rec.*, 1933-34, **58**, 349.

[2] Van Slyke, D. D., Hiller, A., Miller, B. F., *Am. J. Physiol.*, 1935, **113**, 611.

[3] *Ibid.*, personal communication from Gersh to the above authors, of p. 613.

these findings have been confirmed by clearance methods.[2] The technic as originally described has been slightly modified and found to be of value in investigating renal function in experimental kidney lesions in dogs and rabbits.

Method. The animal is given intravenously 5 cc per kg of a 10% solution of sodium ferrocyanide [$Na_4Fe(CN)_6 \cdot 10H_2O$]. This solution is injected rapidly. After an interval of approximately 5 minutes, or as soon after the completion of the injection as ferrocyanide is detected in the bladder washings (it may be detected almost immediately after the initiation of the injection in a normal dog, or it may not appear at all, if there is severe renal damage), the animal is lightly anesthetized with intravenous sodium pentothal (15 mg per kg) and the left kidney exposed by a subcostal flank incision, but not manipulated in any way. A second intravenous injection of sodium ferrocyanide solution is given in the same dosage as the first and, simultaneously with its completion, the pedicle of the exposed kidney is clamped and cut and the kidney removed. Sections 1-2 mm thick are immediately cut and, supported on squares of copper screen, are dropped into a beaker of hexane which has been cooled to approximately −80°C by immersion in a bath of solid CO_2 in methyl-cellosolve. After a few minutes the frozen kidney slices are transferred to a dry flask chilled to −80°C. This is then connected to the manifold of a lyophilizing apparatus and the tissues are dehydrated *in vacuo* for 24 hours. The temperature of the flask containing the sections is maintained at the beginning of the dehydration at −25° to −35°C by a bath of equal weights of ice and chilled 95% ethyl alcohol in a Dewar flask and during the last 4 hours of this period the flask containing the kidney slices is allowed to come from approximately −10°C to room temperature. Melted paraffin is added to the warmed flask and the dehydrated tissues are infiltrated with paraffin by evacuation of the air in the flask; sections are cut at 25 micra and mounted without contact with water by gently spreading them on a warmed slide. The mounted sections are then exposed (dropping bottle technic) in succession to saturated solutions of ferric chloride in xylol and absolute alcohol and a 2% solution in 95% ethyl alcohol. Rinsing in 95% ethyl alcohol, dehydration in absolute ethyl alcohol, clearing in xylol and mounting in clarite then follows. A duplicate section may be counterstained by alcoholic eosin after having been run through the alcoholic ferric chloride solutions. In examining the sections that are not counterstained, the use of a substage lamp with a pink filter facilitates the detection of small amounts of the blue ferric ferrocyanide precipitate.

Results. A section prepared in this manner from a normally functioning kidney usually shows a granular blue deposit within Bowman's capsule and a blue-staining of the tuft and capsule itself. At times there may be no deposit, but the blue-staining of the capsule indicates that ferro-cyanide has been filtered. The purpose of the second injection of sodium ferrocyanide is to insure that there will be a deposit of ferric ferrocyanide within Bowman's space; often none can be found here in a kidney with normal function after only one injection, as recommended by Gersh. A blue deposit is detectable within the lumen of the proximal convoluted tubules, or adherent to the margin of their cells. (These tubules often may be somewhat distorted by artefact.) In the remainder of the tubular system the deposit in the lumen is heavier than in the proximal convoluted tubules.

Various experimentally produced renal disturbances have been studied by the use of this method. Acute hydronephrosis was produced by ligation of one of the ureters and the kidneys studied 24 hours later. In the hydronephrotic kidney, ferrocyanide precipitate was plentiful in the glomerular spaces and in most of the proximal convoluted tubules, scanty and only irregularly present in the loops of Henle and distal convoluted tubules and usually absent in the collecting tubules. In the control kidney the precipitate was found throughout the tubular system.

In a dog that had received 3.5 mg per kg of $HgCl_2$ 24 hours previously, the ferrocyanide deposit was plentiful in the glomerular spaces. The necrotic cells of the proximal convoluted tubules were stained diffusely blue but no

precipitate was found in the rest of the tubular system.

The method has also been used in the study of the renal injury produced by intravascular hemolysis and by the injection of solutions of hemoglobin. These results will be reported in detail subsequently.

Summary. Gersh's ferrocyanide histochemical renal function technic, slightly modified, has been found useful in studying experimentally produced renal lesions. In contrast to the finding of Gersh that such preparations fade rapidly, these have shown no change after 9 months.

15520

A Dialyzable Medium for Cultivation of Group A Hemolytic Streptococci.*

VINCENT P. DOLE. (Introduced by Homer F. Swift.)

From the Hospital of the Rockefeller Institute for Medical Research, New York City.

In the course of work with the filtrates of streptococcal cultures a need was felt for a purified medium capable of supporting abundant growth of the organisms. Synthetic media of the present day did not appear to meet the requirements fully since no one of these media is uniformly capable of supporting heavy growth of all pathogenic strains of interest. Ultimately such defined media should be sufficiently developed to supplant the procedures described in this report.

Since it had previously been shown by Stock[1] that some toxin-producing streptococci are capable of growing on a medium containing only dialyzable constituents, a trial was made with heart muscle extract and commercial peptone purified by preliminary dialysis. It was found that the medium made with these purified constituents actually allowed more rapid and abundant growth in most cases than the standard Todd-Hewitt broth[2] of similar composition. The success of these efforts suggested that the medium might find a wider use than had originally been contemplated.

Preparation of the Purified Beef Heart Extract. Beef heart, after being stripped of fat and finely ground, is soaked overnight at 4°C in water,† 250 cc per pound of meat. Following this infusion the mixture is heated to 85°C for 30 minutes. This amount of heating appears to be just sufficient to coagulate muscle proteins and to express intracellular fluid, yet sufficiently mild to spare most of the heat-labile growth factors. A ten-fold reduction in the quantity of meat required is made possible by this minimal exposure to heat; with more violent extraction or with subsequent heat sterilization of the completed medium, a larger quantity of meat is needed.

At the end of the 30-minute heating period, the extract is filtered through fluted filter paper (supported under the point of the cone with cheese-cloth) and the juice expressed with a wooden masher, then cooled by immersion of the receiving flask in running water. The pink, slightly turbid filtrate, now about 300 cc per pound of meat, is introduced into cellophane casings‡ each about one meter in length and suspended in the upper two-thirds of a suitable tall, narrow vessel against 2 vol-

* This investigation was carried out under a contract between the Rockefeller Institute for Medical Research and the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 Stock, A. H., *J. Immunol.*, 1939, **36**, 489; *J. Biol. Chem.*, 1942, **142**, 777.

2 Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35**, 973.

† No difference in the rate or amount of growth was observed when New York City tap water was compared with distilled water.

‡ Visking Cellulose Sausage Casing, "NoJax" 27/32.

TABLE I.
Comparison of Commercial Peptones.

Brand	% dialyzable	pH of dialysate	Order of increasing color of dialysate	Precipitate with phosphate	Order of decreasing value for growth	Cost per lb
Pfanstiehl	79	5.32	9 (B)	±	1	$4.10
Difco proteose	63	6.80	3 (Y)	±	2	6.00
" neopeptone	80	6.90	7 (B)	++	3	6.00
" bactopeptone	89	7.28	4 (Y)	0	4	4.85
" tryptose	78	7.15	5 (Y)	0	5	6.00
" tryptone	85	7.18	6 (Y)	0	6	4.50
Wilson bacteriological	89	5.57	2 (Y)	0	7	1.10
Witte	55	6 70	1 (Y)	++	8	*
Fairchild	85	5.43	8 (B)	0	9	*

B—Brown. Y—Yellow. *Not available.

umes of water outside. After 12-18 hours dialysis at 4°C, the outside water is replaced; this in turn is replaced after a similar interval for a third dialysis. In this way an estimated 90% of the dialyzable components are extracted into a volume six times the volume of the original filtrate.

If the pooled dialysate is not to be used immediately for media, it should next be concentrated *in vacuo* (15-20 mm Hg) to about 1/20 the volume. With care it may be possible to avoid the use of an antifoaming agent in this process; if one is required, it appears preferable to make small additions of ethyl alcohol rather than the more toxic and persistent octyl alcohol customarily employed.

On standing overnight in the ice box, this concentrate yields an abundant crop of creatine and creatinine crystals which may be removed by centrifugation and discarded. No difference could be detected in the quantity of growth supported by the medium when these crystals were eliminated from one portion and retained in another.

The concentrate may be stored for a period of at least eight months, or longer, by freezing in plastic containers in a dry ice box or by freezing and drying in high vacuum. The latter procedure appears to be the one of choice, despite some difficulty in the drying due to the high salt content with resultant hygroscopic nature, since portions of the dried material may readily be weighed to meet the varied requirements of different experiments.

A yield of about 8 g of the dried extract per 454 g (1 lb) of the original meat is obtained. Excellent growth is supported by 1 g of this material per liter of medium, or with frozen preparations, a quantity representing 1/8 lb of meat per liter. These quantities are at least twice the minimum requirements for optimal growth.

Purification of the Peptone. In Table I are listed various properties of different commercial peptones. It will be seen that there are wide differences in their capacities to support growth, in cost and in color, so that in any particular problem the choice must be governed by the relative weight assigned to these factors. It has, moreover, been observed (Fig. 1) that the different peptones cannot be consistently graded on the basis of growth rate and yield since their relative values in this regard are in part dependent on the particular strain of group A streptococcus employed.

An experiment shown in Fig. 1 measured turbidimetrically the growth of 5 strains of group A hemolytic streptococcus in dialysate media made with 9 different commercial peptones. At zero time each tube, containing 9 cc of medium, was inoculated with one loop of a 12-hour broth culture of organisms. Data are shown for the dialysate media (1 to 9) and for the stock Todd-Hewitt medium[2] made with unpurified Pfanstiehl peptone.

Of the peptones studied the 2 most useful for present purposes appear to be Pfanstiehl peptone and Difco Proteose. Both support excellent growth for all strains tested. The lower cost of the former recommends it for large scale uses in which economy is a more important consideration than freedom from colored impurities. These colored ma-

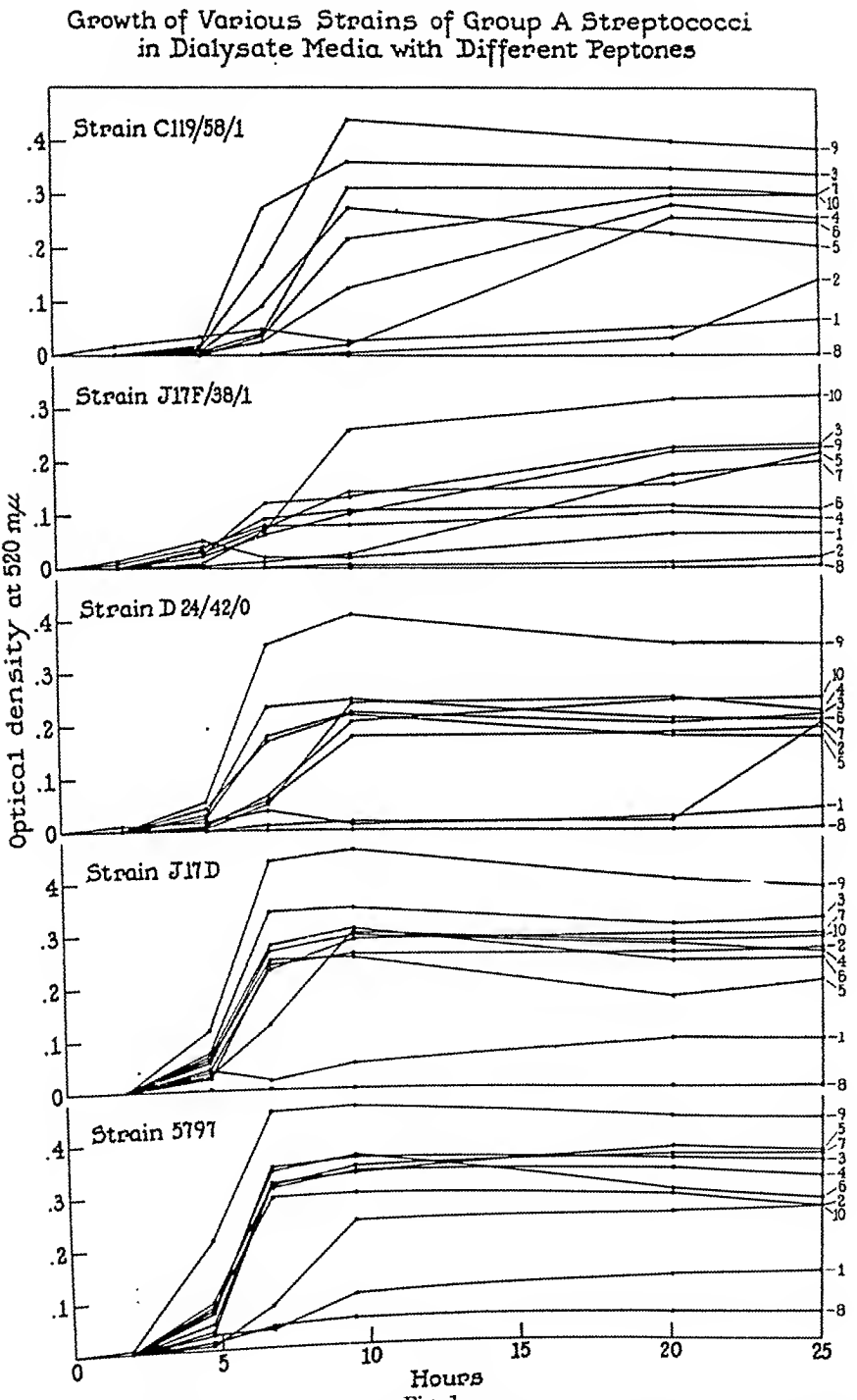

Fig. 1.

Growth of 5 different strains of group A hemolytic streptococci in dialysate media made with the following peptones: 1, Witte; 2, Wilson bacteriological; 3, Difco proteose; 4, Difco bactopeptone; 5, Difco tryptose; 6, Difco tryptone; 7, Difco neopeptone; 8, Fairchild; 9, Pfanstiehl; 10, Stock Todd-Hewitt medium with unpurified Pfanstiehl peptone.

terials can be reduced, but not eliminated, by repeated adsorption with charcoal at different pH's; what remain tend to persist through any subsequent fractionations of the culture fluid and may for this reason be found most objectionable. Proteose peptone, on the other hand, is initially of low color content and can be rendered almost colorless by charcoal adsorption of a 20% solution at pH 8.0. This step also removes the slight precipitate that sometimes develops at this alkalinity.

As a consequence of the high calcium content of neopeptone (Difco), the dialysate medium with this peptone becomes turbid after addition of the phosphate. If removed by preliminary filtration, the turbidity reappears during growth.

For purification the peptone is dissolved in water at 80°C to make a 20% solution, to which activated charcoal about 50 g per liter is added; the mixture is held at 80°C with stirring for one hour, then filtered hot through soft filter paper with the aid of suction. The clear filtrate is then dialyzed in a manner similar to that of beef heart extract.

After completion of dialysis, the pooled dialysate is adjusted to pH 8.0 and again adsorbed with charcoal 50 g per liter, overnight at 4°C. Following removal of the charcoal by filtration, the solution may be concentrated for storage by evaporation *in vacuo*, followed by freezing and drying. It is desirable to prepare as large a quantity of the dried purified peptone as possible in order to assure uniformity of experimental results. There appear to be uncontrolled variations in the qualities of different lots of the same brand of commercial peptone.

Preparation of the Complete Medium (Solid Content 2.3%). The following ingredients per liter of ultimate volume are dissolved in about 900 cc of water, adjusted to pH 8.0 with approximately N/1 NaOH, and then brought to a volume of 950 cc.:

Peptone: 15 g (or the dialysate from 20 g of original peptone)

NaCl: 2 g

Na_2HPO_4 (anhydrous): 0.5 g

This solution may be heat sterilized without impairment of the growth properties, although some undesired color and precipitate may be formed.

Just before use, the medium is completed by addition of 50 cc per liter of final volume of the following mixture, sterilized by filtration:

Beef heart extract: 1 g (or the dialysate from 1/8 pound of meat)

Glucose: 2 g

(Volume adjusted to 50 cc, pH to 8.0 at this point)

$NaHCO_3$: 2 g

Since the whole medium filters at about the same rate as water, it might be preferred to sterilize entirely by filtration. In this case, all ingredients are dissolved in their final concentration (the bicarbonate again being withheld until the pH is adjusted to 8.0) and the lot filtered.

In either case the medium should be inoculated immediately after completion; otherwise the pH may rise to 8.5 or higher in a few hours from decomposition of the bicarbonate. If delay in inoculation is unavoidable, the bicarbonate should be omitted and either added later or the pH during growth controlled by intermittent additions of 18 N NaOH.

It has been found possible, although not for our purposes advantageous, to combine the dried materials in the proper proportions so that complete medium may be formed merely by the addition of water. Such a procedure may be useful when it is necessary to use small amounts of a uniform medium at irregular intervals.

Discussion. Strains C119/58/1 and J17F/38/1, shown in Fig. 1, were selected for study because they had been found by Pappenheimer[3] to be the 2 most difficult to cultivate in the defined medium of Bernheimer, Gillman, Hottle and Pappenheimer.[4] Strains D24/42/0 and J17D were included because they had been found by Wilson[5] to require xanthine. Strain 5797 was currently in use for other purposes and was known to

[3] Pappenheimer, A. M., personal communication.

[4] Bernheimer, A. W., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., *J. Bact.*, 1942, **43**, 495.

[5] Wilson, A. T., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 249.

grow abundantly on stock media. With all 5 of these strains Hartman[6] obtained light growth in the partially defined medium of Adams and Roe[7] which, following Wilson,[5] had been supplemented with horse serum and xanthine.

The dialysate medium evidently contains growth factors not present in sufficient concentrations in either of these semisynthetic media. No attempt has been made to fractionate the natural products further to elucidate the difference.

A potential advantage for the dialysate medium is the reduction in antigenic materials otherwise introduced with unpurified peptone and beef heart. It was found by Todd[8] that horse serum containing strong precipitating antibodies for some antigens in Proteose peptone failed to show any reaction with the corresponding dialysate medium.

Summary. A procedure is described for preparation of a medium containing only 2.3% solid material, all dialyzable. The medium has been found uniformly capable of supporting rapid and heavy growth of group A hemolytic streptococci.

The author is indebted to Dr. Rebecca C. Lancefield and to Miss Dorothy Sloan for their criticism and help.

6 Hartman, T., personal communication.

7 Adams, M. H., and Roe, A. S., *J. Bact.*, 1945, **49**, 401.

8 Todd, E. W., personal communication.

15521

Serum Levels and Excretion Studies in Mice Following Injection of a Penicillin-Albumin Complex.

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In a previous paper[1] mention was made of the fact that penicillin administered intramuscularly to mice in the form of a penicillin-albumin complex was eliminated more slowly than solutions of penicillin in water. Since the publications by Romansky and Rittman[2,3] have shown that penicillin suspended in peanut oil is excreted more slowly than solutions in saline and that when beeswax is added, in concentrations by volume ranging from 0.75 to 6%, the rate of excretion is further reduced, it seemed of interest to compare the efficacy of a penicillin-albumin complex with suspensions of penicillin in oil and in oil-beeswax.

Method. In the studies to be described comparison of serum levels and rates of excretion was made following intramuscular injection of an aqueous solution of a penicillin-albumin complex, suspensions of a calcium salt of penicillin in peanut oil and in peanut oil containing 2, 3 and 5% beeswax* and solutions of penicillin in distilled water.

Since earlier studies on rabbits had shown that serum levels and excretion rates on individual animals frequently varied widely, pooled serum and urine from groups instead of from individual animals were used. The method consisted of the intramuscular injection of penicillin in volumes from 0.2 to 0.5 ml into groups of 30 to 80 mice. The dosage in different experiments ranged from 20 to 400 Oxford units per 20 g mouse. For the excretion studies the first urine col-

1 Chow, B. F., and McKee, C. M., *Science*, 1945, **101**, 67.

2 Romansky, M. J., and Rittman, G. E., *Science*, 1944, **100**, 196.

3 Romansky, M. J., and Rittman, G. E., *Bull. U. S. Army Med. Dept.*, 1944, No. 81, 43.

* These suspensions were kindly supplied by Dr. A. E. Jurist of Products Development Laboratories of E. R. Squibb & Sons. The suspensions were made on a weight volume basis from commercial penicillin.

lection was usually made one-half hour after injection and the second one-half hour later. Thereafter, collections were made at hourly intervals over periods of time ranging from 7 to 14 hours depending upon the nature of the preparation and the dosage given. Each hourly sample consisted of the pooled urine from the total number of mice in the group. The mice were picked up one at a time and the urine collected in a test tube. Urination was usually spontaneous but gentle pressure on the abdomen aided excretion. No special effort was made to empty the bladders of the mice before beginning the experiment. However, handling of the mouse during injection usually caused urination. Early samples were diluted 1-10 with distilled water and passed through Swinney (Seitz type) filters. Later samples were filtered undiluted.

For determination of penicillin concentrations the urine samples were further suitably diluted and tested, first by a 2-fold serial dilution test to determine the approximate concentration, and later by a more precise test in which small amounts (0.1 to 0.03 ml) of suitable dilutions were added to tubes containing 2 ml of a 10^{-6} dilution of a *Staphylococcus aureus* (Heatley) culture. Readings were made on the basis of turbidity after incubation for 18 hours at 37°C, the end point being the highest dilution showing complete inhibition. If, however, there were dilutions showing partial inhibition, the end point was considered to be midway between those showing complete and those showing partial inhibition. The potency was determined by comparison with a standard solution of crystalline penicillin G. By the 2-fold serial dilution test it was possible to determine as little as 0.04 units per ml, but by the more precise test it was not possible to show amounts less than 0.15 to 0.2 units per ml. The highest concentration tested by this method was a 1-20 dilution, while by the serial dilution test it was 1-4.

In the serum level studies the same time intervals for collection were used as in the excretion studies. Five or 6 mice were bled to death from the heart at each time interval and the samples of blood pooled. One-half

TABLE 1.

Comparative Rate of Excretion of Penicillin in Urine of Mice Following Intramuscular Injection of a Penicillin-albumin Complex, a Suspension of Penicillin in Peanut Oil, and Solutions of Penicillin in Water.

Hr. after injection	Penicillin-Albumin complex				Cal. salt of penicillin in oil				Crystalline penicillin G aqueous sol. Cal. salt of penicillin aqueous sol.							
	Vol. urine, ml	O.U./ml urine	Total O.U.	%	Vol. urine, ml	O.U./ml urine	Total O.U.	%	Vol. urine, ml	O.U./ml urine	Total O.U.	%	Vol. urine, ml	O.U./ml urine	Total O.U.	%
½	3.3	44	145	19.3	0.85	154	131	17.5	0.35	1076	377	50.3	0.8	519	415	55.3
1	1.2	75	90		0.5	144	72		0.4	218	82.7		0.4	97	38.8	
2	1.6	55	88		1.2	46	55		1.8	22	39.6		2.4	5.6	13.4	
3	.9	39	35		1.2	12.8	15.4		1.3	1.58	2		1.7	0.88	1.5	
4	1.0	21	21		1.3	4.0	5.2		1.4	0.21	0.3		1.7	0.15	0.26	
5	1.0	11.3	11.3		1.0	1.2	1.2		1.7	0.1	0.17		1.4	0.08	0.11	
6	1.2	4.2	5		1.35	0.48	0.65		2.0	0.08	0.16		1.3	0.04	0.05	
7	.9	2.0	1.8		1.4	0.24	0.34		1.2	<0.03			2.0	<0.03	0.06	
			397.1	63			280.79	37.4			506.43	67.5			469.18	62.5

Thirty mice in each group received doses of 25 Oxford units per mouse.

to three-quarters ml could be obtained from each mouse. Blood was collected over periods of 4 to 10 hours depending upon the expected concentration of penicillin. The blood was allowed to clot, was then centrifuged, and the clear serum collected for test. Penicillin assay on the serum samples was done in the same manner as on the urine samples.

The penicillin-albumin complex was made by equilibrating a solution of crystalline penicillin G with human albumin as described by Chow and McKee.[1]

Results. In the first experiments, 4 groups of 30 mice received intramuscular injections of 25 Oxford units per mouse of a penicillin-albumin complex, of a calcium salt of penicillin in peanut oil, of solutions in water of crystalline penicillin G or of a calcium salt of penicillin. Urine was collected over a period of 7 hours and penicillin determinations made. No investigation of serum levels was made. The results are shown in Table I.

From the 2nd to the 7th hour higher concentrations of penicillin were present in the urine of those mice which received the penicillin-albumin complex and the penicillin in oil than in those which received solutions in water. The reverse was true one-half hour after injection. In those mice which had received penicillin in water approximately one-half of the total amount of penicillin injected was excreted during the first half hour.

In experiments with mice it is not possible to collect the total amount of urine excreted but by making frequent collections there is probably little loss. In Table I the volumes collected at the different time intervals and the total excretion in Oxford units is given. When aqueous solutions of penicillin were used and excretion was rapid the total recoveries in the 2 experiments were 67.5 and 62.5%. When a penicillin-albumin complex was used the total recovery was 53%, and when a calcium salt in oil was used recovery was 37.4%. In another experiment, not shown in Table I, in which 400 Oxford units in a 5% beeswax-oil mixture were injected, and excretion was greatly retarded, the total recovery was only 13.7%. It would seem that total recovery of penicillin was reduced as elimination from the body was retarded.

In further experiments a penicillin-albumin complex in 4 different dose levels, 25, 100, 200 and 400 Oxford units per mouse, was compared with like doses of aqueous solutions of penicillin. In all cases the penicillin-albumin complex retarded elimination. In general, like concentrations of penicillin were present in the urine 3 to 4 hours later in those mice injected with the penicillin-albumin complex than in those receiving aqueous solutions of penicillin.

A series of experiments was carried out in which a penicillin-albumin complex was compared with a calcium salt of penicillin in water, in peanut oil, and in peanut oil containing 2, 3 and 5% beeswax. Comparative serum levels and excretion rates were obtained following injection of 400 Oxford units per mouse. All results are expressed in units per ml of serum or urine.

In 2 out of 3 experiments with aqueous solutions, no penicillin was demonstrated in the serum 2 hours after injection; in the third experiment 0.06 unit was still present at this time but none one hour later. With the calcium salt suspension in peanut oil, penicillin (0.33 and 0.17 unit) was still demonstrable in the serum in 2 out of 3 groups at 4 hours; later tests were not made. With the 2% beeswax-oil, the last test was made 5 hours after injection, at which time the serum showed 0.39 unit. With the 3% beeswax-oil, the last test made at 10 hours showed 0.1 unit, and with the 5% beeswax-oil there was 0.17 unit at 9 hours and a trace at 13 hours. With the penicillin-albumin complex, 0.036 unit was present at 5 hours but none at 6 hours. Since it is generally agreed that less than 0.05 unit per ml is an effective inhibiting serum level, neither the oil nor the oil-beeswax experiments were carried out for a sufficient length of time. It would seem, however, that the penicillin-albumin complex was less effective even than 2% beeswax in oil in prolonging effective serum levels.

The figures on excretion indicate that the retarding effect of the penicillin-albumin complex was about the same as that of penicillin in oil. When beeswax was added to

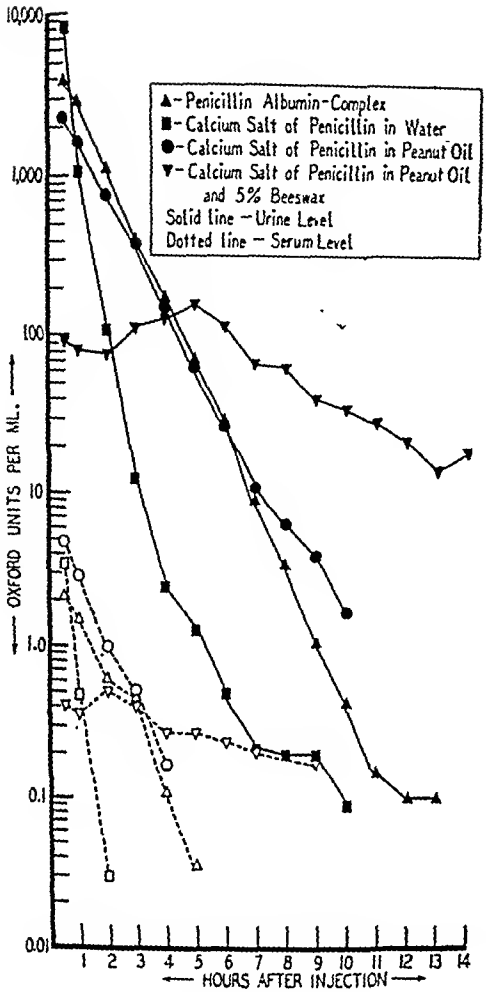

Fig. 1.

the oil the excretion rate was still further retarded, the degree of retardation being increased as the per cent of beeswax was raised. With the penicillin-albumin complex, the suspensions in oil, and solutions in water, the peak of excretion was reached one-half hour after injection. When 2% beeswax was added to the oil the peak in one experiment was still reached at one-half hour but in another experiment the peak was one hour after injection. With 3 and 5% beeswax the peaks of excretion came 3 to 5 hours respectively after injection. As with the serum level studies, experiments were not continued for a sufficient length of time to observe final elimination of penicillin.

Tests made 10 hours after injection of 400 Oxford units of the various preparations gave the following results in units per ml of urine: aqueous penicillin (3 experiments) 0.18, 0.08 and <0.02, suspension in oil (3 experiments) 3.6, 1.5, and 0.15, 2% beeswax-oil (2 experiments) 11.6 and 9.3, 3% beeswax-oil 19.2, 5% beeswax-oil 36.7, and a penicillin-albumin complex 0.44. Tests made 14 hours after injection of the 2, 3 and 5% beeswax-oil suspensions showed 5.7, 10.2 and 19.8 units per ml respectively and 23 hours after injection of 3 and 5% beeswax-oil suspensions 2.8 and 6.6 units respectively were demonstrated. Thus with both 3 and 5% beeswax in oil, penicillin was demonstrable in the urine 23 hours after injection in larger amounts than at 9 to 10 hours after injection of the penicillin-albumin complex. Fig. 1 shows the similarity of blood levels and excretion rates following injection of a penicillin-albumin complex and a suspension of a calcium salt of penicillin in oil. The more rapid elimination when an aqueous solution was injected and the much greater retention when a suspension in oil containing 5% beeswax was used are also shown. Where several experiments were done with the same preparation the figures were averaged.

The results in mice using suspensions of penicillin in oil and in oil-beeswax are in essential agreement with those of Romansky and Rittman who used rabbits as the experimental animals and extended their investigations to man. Richardson, Miller and Ahlgren,[4] using dogs, found slight retardation of absorption when a penicillin suspension in oil was given subcutaneously. Nelson[5] injected 2 human subjects intravenously with 35,000 and 63,000 units of a penicillin-albumin complex and found no prolongation of the action of penicillin. Intramuscular injection in man has, we believe, not been tried.

Summary. A method is described for the use of groups of mice in serum level and excretion studies on penicillin.

A slight prolongation of penicillin in the body of the mouse resulted from the intra-

[4] Richardson, A. P., Miller, I., and Ahlgren, M. W., unpublished.

[5] Nelson, R. A., personal communication.

muscular administration of a penicillin-albumin complex and a suspension of penicillin in peanut oil. When beeswax in 2, 3 and 5% concentration was added to the penicillin-peanut oil mixture the presence of penicillin in the body was greatly prolonged, the degree of prolongation being greater as the concentration of beeswax was increased.

15522 P

Heterophile Antibody Following Administration of Blood Group-Specific Substances.

CORNELIA A. EDDY, RALPH E. WHEELER, AND LOUIS K. DIAMOND.
(Introduced by David Rapport.)

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In the course of studies on the production of isoagglutinins in human volunteers an agglutinin for sheep cells was encountered in the sera of a number of persons receiving group-specific A substance (Witebsky) and group-specific AB substance (Witebsky).* Twenty-three student volunteers of Tufts College Medical School participated. Sixteen belonging to blood groups B and O received 0.17 to 1.0 mg of A substance intravenously. Blood samples were taken before injection and again 2 weeks after. Sheep cell agglutinins were studied by the method of Paul and Bunnell.[1] An increase in titre of the second serum of 3 tubes or more above the titre of the first was observed in 7 volunteers, 9 remained unchanged or showed less definite increases. Seven volunteers belonging to blood group A received 0.05 mg of AB substance intravenously. Of these only one showed an increase of just 3 tubes.

Since A and AB substances are used to neutralize the isoagglutinins of O blood used for transfusion, the question arose of whether individuals transfused with such blood also developed sheep cell agglutinins. Blood sera were obtained before and one week after transfusion of 15 individuals and compared by the Stuart method.[2] Unlike the student volunteers, the members of this group received A and AB substances combined. Eight belonged to blood groups B and O; of these 5 showed an increase in titre of 3 tubes or more. Seven belonged to blood group A and of these one showed a definite increase.

Volunteers and transfusion recipients whose sheep-cell agglutinin titres were low before injection showed more spectacular rises than those with initially elevated titres. Of 27 individuals, whose original titres were 1/40 or less, 14 showed an increase of 3 tubes or more. Of 11 with original titres above 1/40, only one showed an increase.

The antibody found in these sera differs from the antisheep antibody found in the sera of individuals with infectious mononucleosis in that it can be absorbed with A substance, while the latter cannot. Further attempts to identify this heterophile antibody by absorption with guinea pig kidney and with boiled beef cells have so far yielded equivocal results.

We are indebted to Dr. Ernst Witebsky for valuable advice, and to Miss Lilliau Rodofsky for extensive technical assistance.

* The group-specific AB substance was supplied by Eli Lilly & Company; the purified A substance was supplied by Sharp & Dohme.

1 Bray, W. E., *Synopsis of Clinical Laboratory Methods*, pp. 279-281.

2 *Diagnostic Procedures and Reagents*, 2nd Edition, 1945, pp. 449-456.

15523

Effect of Streptomycin on Experimental Infections Produced in Mice with Streptomycin Resistant Strains of *M. tuberculosis var. Hominis.**

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Youmans, Williston, Feldman and Hinshaw[1] have shown that virulent tubercle bacilli may acquire resistance to the *in vitro* bacteriostatic action of streptomycin, either by prolonged exposure to streptomycin in the test tube, or in patients undergoing treatment with this agent. These findings raise the important questions of whether these streptomycin resistant tubercle bacilli are still pathogenic and, if so, whether they are equally resistant to the bacteriostatic action of streptomycin while producing infection in a susceptible animal.

Methods. Eighty white mice† weighing approximately 25 g each were divided into 4 groups of 20 mice each. The first group was injected intravenously with 0.1 mg of a suspension of a 21-day-old culture of a streptomycin sensitive H37Rv strain. The second group was injected similarly with 0.1 mg of a 21-day-old culture of an H37Rv strain which had become resistant to more than 1000.0 μg of streptomycin per ml following exposure to streptomycin *in vitro* (designated H37RvR). The third group was injected intravenously with 0.1 mg of a cul-

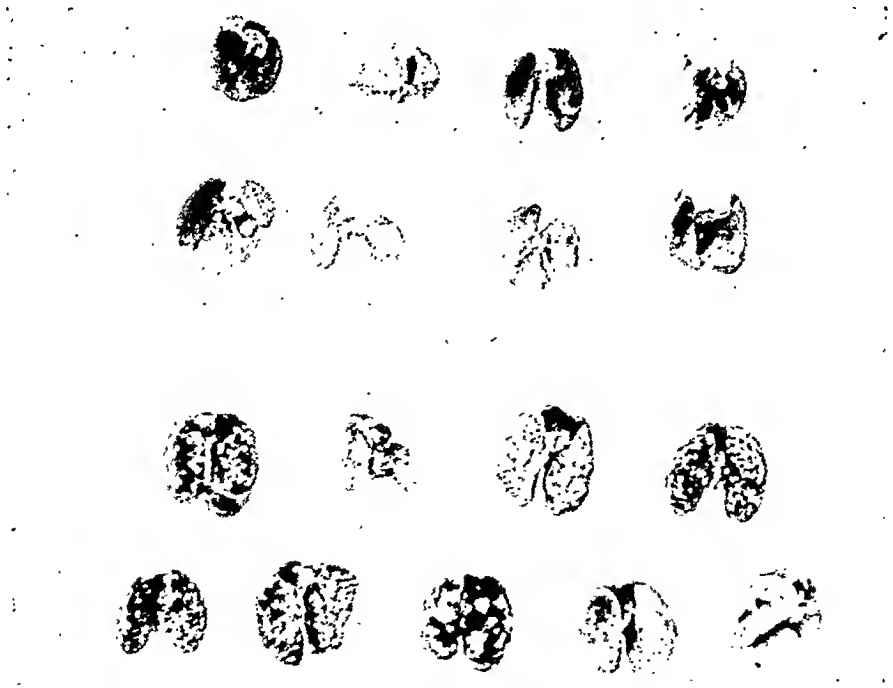

FIG. 1.
Lungs of mice infected with streptomycin sensitive strain H37Rv. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

* This work was aided by a grant from Parke Davis and Company, Detroit, Mich.
The streptomycin was furnished through the courtesy of Dr. L. A. Sweet, Parke, Davis and Company, Detroit, Mich.

[1] Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet. Mayo Clinic*, 1940, 21, 126.

† Strong A strain.

TABLE I.

Effect of Streptomycin in Mice on Streptomycin Sensitive and Streptomycin Resistant Strains of *M. tuberculosis var. hominis.*

Organism	Streptomycin sensitivity *in vitro* (μg/ml)	Amt streptomycin inj. daily (μg)	No. of mice	No. dead	% mortality	Avg amt of gross pulmonary tuberculosis
1. H37Rv	0.78	3000	8*	0	0.0	None
2. H37Rv	0.78	None	10	6	60.0	+++
3. H37RvR	1000.0	3000	10	10	100.0	++++
4. H37RvR	1000.0	None	10	10	100.0	++++
5. 15	0.78	3000	9†	0	0.0	None
6. 15	0.78	None	9†	6	66.0	+++
7. 67	1000.0	3000	10	5	50.0	+++
8. 67	1000.0	None	9†	5	44.0	+++

* Two mice died day after injection.
† One mouse died day after injection.

ture isolated from a patient with pulmonary tuberculosis (strain 15). The growth of this culture was inhibited *in vitro* by less than 1.0 μg of streptomycin per ml. The fourth group was injected intravenously with 0.1 mg of a culture isolated from the same patient after several months treatment with streptomycin (strain 67). At this time this culture grew readily *in vitro* in a concentration of streptomycin of 1000.0 μg per ml.

Half of the mice in each group served as controls whereas the other 10 mice were

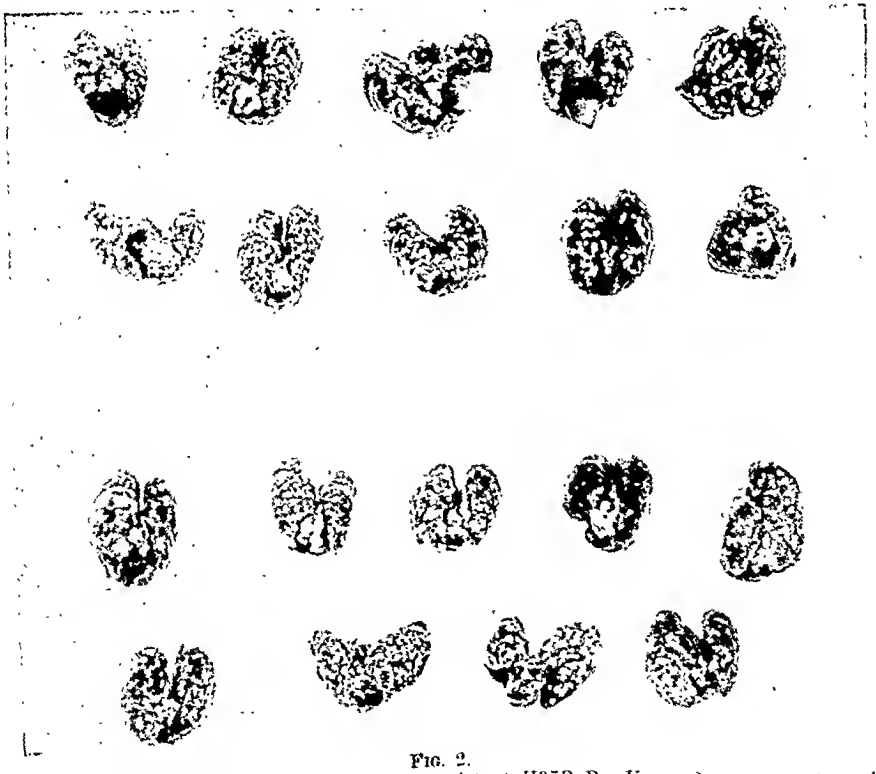

FIG. 2.

Lungs of mice infected with streptomycin resistant H37RvR. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

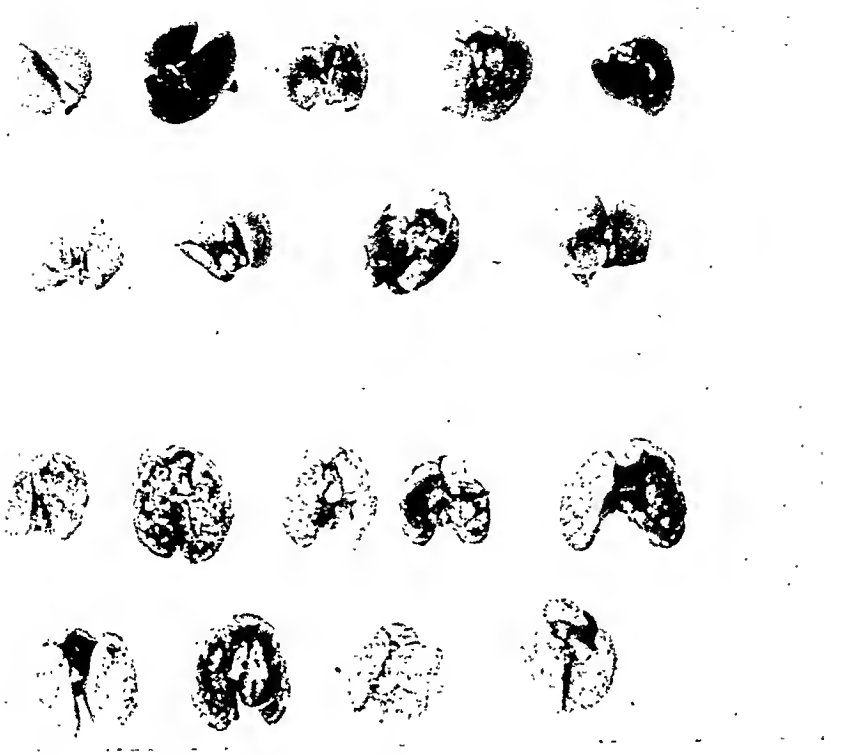

FIG. 3.
Lungs of mice infected with streptomycin sensitive strain 15. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

treated with a total of 3000.0 μg of streptomycin daily, given subcutaneously in 4 equally divided doses at intervals of 6 hours. Treatment was continued for a period of 35 days at which time the surviving mice were sacrificed and observed for the presence of gross tuberculous lesions. Portions of the lungs of 2 mice in each group were cultured for the presence of tubercle bacilli and the cultures obtained were retested for their sensitivity to streptomycin in the manner described previously.[2]

Results. Table I shows the results of the treatment of mice experimentally infected with streptomycin sensitive and resistant cultures of virulent human-type tubercle bacilli. Groups 1 and 5 confirm our previous report[3] that streptomycin exerts a marked suppressive effect on tuberculous infections of mice. All the mice in these 2 groups gained weight over the period of treatment and showed no gross evidence of tuberculosis upon autopsy. Groups 3, 4, 7 and 8 show clearly that not only are the streptomycin resistant cultures virulent for mice but also that streptomycin in the doses used does not influence the course of the infection.

Fig. 1, 2, 3 and 4 show the lungs of the mice. These illustrate graphically the effect of streptomycin in suppressing the infections produced by the streptomycin sensitive tubercle bacilli and the ineffectiveness of streptomycin for the suppression of infections caused by the streptomycin resistant strains.

Tubercle bacilli were reisolated in cultures from the lungs of mice from all 10 groups, though in smaller numbers from those infected with the streptomycin sensitive cultures and treated with streptomycin. The streptomycin

[2] Youmans, G. P., *Quart. Bull. North. Univ. Med. School*, 1945, **19**, 207.

[3] Youmans, G. P., and McCarter, J. C., *Am. Rev. Tuberc.*, 1945, **52**, 432.

FIG. 4.

Lungs of mice infected with streptomycin resistant strain 67. Upper 2 rows, streptomycin treated. Lower 2 rows, no treatment.

sensitivity of these reisolated cultures was found to be the same as before injection into the mice.

Summary. Streptomycin resistant human type tubercle bacilli were found to be as virulent for white mice as streptomycin sensitive strains. Infection produced in mice with these streptomycin resistant cultures was not suppressed by treatment of the mice with streptomycin.

15524

Variation in Influenza Viruses. A Study of Heat Stability of the Red Cell Agglutinating Factor.*

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For estimating the degree of stability of a variety of materials, both biological and non-biological, it is common practice to utilize elevated temperatures in order to accelerate

* These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

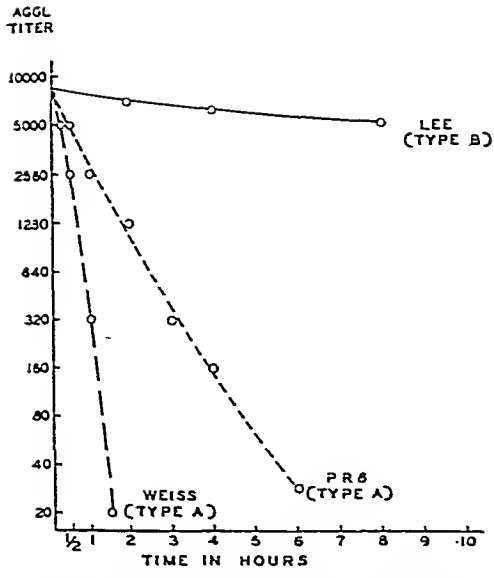

FIG. 1.

Curves showing comparative rates of inactivation at 61.5°C of Lee, PRS, and Weiss strains of influenza virus in untreated allantoic fluids.

the processes that occur slowly at normal temperatures. In view of the slow deterioration at temperatures up to 37°C of the immunizing and hemagglutinating antigens of influenza virus,[1-5] it seemed desirable to define a convenient temperature at which observations could be made rapidly in order to facilitate studies of the relative stability of different preparations of virus or vaccine. In the course of preliminary experiments at 56-65°C it was found that distinct differences exist among strains in terms of the rate of destruction of the red cell agglutinating capacity of the virus. These findings were explored further. It is the purpose of this preliminary communication to present the evidence obtained thus far revealing the existence of wide differences in the stability of hemagglutinin not only among strains but among different lines of the same strain as well.

Materials. Various strains were compared and in some instances the same strain which had been passaged in different hosts, or culture media, was examined. The strains and passage histories will be indicated in the text. The studies reported here have been done on allantoic fluid obtained without red blood cells from infected chick embryos.

Methods. As the source of heat, a water bath was used in which the temperature was accurately controlled within less than 0.1° in the region of 61.5°C This temperature was employed simply because the mercury thermo-regulator was originally set at this temperature when an attempt was made to adjust it to 62°C. Preliminary tests indicated that 62°C was convenient for the majority of strains examined. In some experiments at lower temperatures less well controlled heating equipment was used. For determining stability to heat, the virus suspensions were distributed in 0.5 cc quantities in small rubber-stoppered test tubes and placed in a rack suspended in the bath. At intervals, a tube containing the 0.5 cc aliquot was removed and immersed in iced water. All samples of the same virus suspension were stored in iced water or in the 4°C refrigerator and tested for titer of hemagglutinin at the same time.

The method for estimating hemagglutinating potency has been described.[6] The technic employed involves the use of 0.5 cc quantities of the serially diluted test material and an equal volume of 0.25% suspension of chicken erythrocytes. Serial dilutions were made with an automatic syringe. The endpoint of the titrations is taken to be the highest dilution showing complete agglutination as revealed by the pattern of the sedimented cells. Titers are expressed as the reciprocal of the final dilution of starting material. Procedures providing greater accuracy were not required since gross changes, rather than slight variations, were expected.

Results. The initial observations were made in the course of experiments with the 3 strains of influenza virus which were present in the vaccine recently used by the Army,[3] *i.e.*, the PRS and Weiss strains of Type A virus and

[1] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

[2] Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1.

[3] Salk, J. E., Pearson, H. E., Brown, P. N., Smythe, C. J., and Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 307.

[4] Miller, G. L., *J. Exp. Med.*, 1944, **80**, 507.

[5] Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

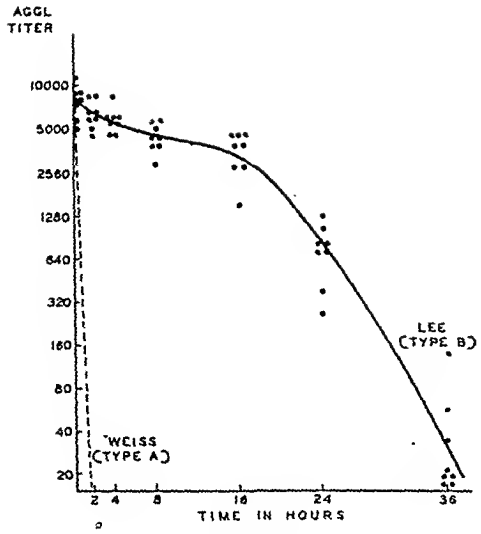

FIG. 2.

Curves showing rates of inactivation at 61.5°C of hemagglutinin of Lee strain of influenza virus Type B in 8 different samples of allantoic fluid and of Weiss strain of Type A virus in 12 different samples of allantoic fluid. (Degree of variation in 8 batches shown on Lee curve. Similar degree of variation along Weiss curve not shown.)

the Lee strain of Type B virus. The PR8 strain had previously been passed through a small number of ferrets, then through 593 mouse transfers, from which it was then introduced into the chick embryo for almost 50 passages. The Weiss strain had come through 3 ferret passages, 32 mouse transfers and 53 egg passages. The Lee strain, after about 8 ferret passages followed by 137 mouse passages, was in the 85th to 90th egg transfer. Fig. 1 shows the results of a typical experiment in which it is seen that the hemagglutinin of the Lee strain was much more stable than those of the PR8 and Weiss strains, and that Weiss was much less stable than PR8. That these variations are related to the different strains of virus and not to individual variations among different preparations of allantoic fluid is suggested by the data shown in Fig. 2. In this experiment 8 different pools of allantoic fluid containing the Lee strain and 12 different pools containing the Weiss strain were compared. While all 12 samples of the Weiss strain had no detectable hemagglutinin after 90 minutes at 61.5°C, little or no change in titer was observed among the 8 samples of the Lee strain in 2 hours. In fact, hemagglutinin of the Lee strain was still detectable after 24 hours, and in some samples after 36 hours.

The difference between the strains illustrated has been observed repeatedly and consistently with successive passages of these particular lines of the respective strains. That the differences are due to properties of the virus and not to some extraneous factor in the allantoic fluid is indicated by examination of the rate of inactivation of the hemagglutinin present in a mixture of the Weiss and Lee strains in allantoic fluid. The admixture with allantoic fluid containing the Weiss strain did not alter the shape of the curve of inactivation of the hemagglutinin of the Lee component. The difference between strains was still evident in preparations in which virus was separated from allantoic fluid by adsorption on red cells and elution[1,7] into a medium other than allantoic fluid, or after adsorption on calcium phosphate[8] and resuspension in physiological solutions. The influence of the composition of the physiological solution on stability will be described elsewhere.

In view of the striking difference in behavior of the Lee strain of Type B virus when compared with the PR8 and Weiss strains of Type A virus, it seemed of interest to determine whether the greater stability of the hemagglutinin was a characteristic of Type B strains. Accordingly, a variety of both A and B strains in allantoic fluid was examined.

A group of 8 Type A strains from different epidemics of the past several years was tested. The prior history of laboratory passage varied, but in all instances the strains exhibited less stability than the PR8 and Weiss strains. Since 61.5°C seemed to be above the critical zone of destruction of the hemagglutinin of these strains, preliminary tests were conducted at 50°C. At this temperature strain difference was evident.

Only 1 of 19 Type B strains tested exhibited a stability curve approximating that of the

[7] Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

[8] Salk, J. E., *Science*, 1945, **101**, 122.

Lee strain. The exception was the "BON" strain isolated in Dr. Burnet's laboratory in Australia in 1943.[9] Of the other 18 strains, all of which had been isolated in chick embryos in this laboratory, two† were obtained from a localized outbreak in May 1945 and the remainder‡ during the epidemic of 1945-46.[10] The 2 strains from the May 1945 outbreak had been passed 30 times in eggs and all of the 16 strains from the 1945-46 epidemic had been passed less than 10 times. Strain difference was again encountered. The hemagglutinin of all but 3 strains was destroyed in 5 to 15 minutes at 61.5°C. The 3 exceptions (Allen, Goodloe and Hacker), which were more resistant, were among the 16 strains obtained in the 1945-46 epidemic. Although the 2 strains isolated from the May 1945 outbreak had been passed 30 times in eggs, they were no more stable than the majority of the newer strains which had had fewer passages. It would appear from these observations that the particular line of the Lee strain, the stability of which has been described, cannot be considered representative of all Type B strains. Moreover, differences exist in the stability of the hemagglutinating property of strains of virus isolated from the same outbreak.

Since the Lee, PR8 and Weiss strains have had long lines of laboratory passage, it seemed possible that the difference between these and the recent strains might be related to this factor, in part at least. Accordingly, the Lee and PR8 strains of virus which had been maintained in different hosts for varying numbers of passages were transferred to eggs and the allantoic fluids obtained were then tested for stability of their hemagglutinating capacity. Tables I and II show the results of 2 such experiments. From these data it is seen that differences exist in the degree of stability of the hemagglutinin of the different lines of the same strain. In view of the greater stability of preparations having the longest history of egg passage, regardless of previous passage in other media, the following was done to determine the influence of change in host on the stability of the hemagglutinating property of the viruses. The regular egg-passage lines of the PR8 and Lee strains were transferred to mice and carried through 14 passages. After successive passages in mice the virus was returned to the egg and tests were made of the stability of hemagglutinin in the initial egg transfer from mouse material. In the series thus far, passage of the egg-line in mice has not altered the stability of the hemagglutinin of the respective strains of virus. The reverse of this experiment, *i.e.*, passage of the ferret or mouse lines through 27 egg transfers, has not changed the character of the virus in terms of the heat stability of the hemagglutinin.

The persistence of line difference, even after change in host or culture medium, suggests that the host factor is probably of secondary importance and that intrinsic differences of an inheritable nature are involved. The following experiment is cited in further support of the suggestion that the viruses maintained in the separate lines are distinct in terms of heat stability of the hemagglutinin. Chick embryos were inoculated with mixtures of the standard egg-line (Table II, Col. 1) and the ferret egg-line (Table II, Col. 3), combined in varied proportions. Examination of the infected allantoic fluids for heat stability of hemagglutinin indicates that viruses of both lines grew simultaneously and were present in different concentrations depending upon the relative quantities of the respective lines of virus in the different inocula.

Reference has been made to results of other studies that have a bearing on the observations reported here. In Hirst's studies,[1] in which he found that infectivity was destroyed more rapidly than hemagglutinating activity, the PR8 and Lee strains did not differ in the rate at which hemagglutinin was destroyed at 55 and 60 C. Miller[4] followed the rate of decline of infectivity and hemagglutinating

[9] Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 1.

† May, 1945, strains—Chaddick and Ector.

‡ 1945-46 strains—Allen, Amdall, Baker, De-Simple, Goodloe, Hacker, Meulder, Mindell, Neubert, Olsen, Orlebeck, Peacock, Potter, Sadowski, Skipton, Solomon.

[10] Francis, T., Jr., Salk, J. E., and Brace, Wm. M., *J. Am. Med. Assn.*, 1946, **131**, 275.

TABLE I.

Effect of Exposure to Temperature of 61.5°C for Varying Intervals on the Hemagglutinin Titers of Allantoic Fluids Containing the PR8 Strain of Influenza Virus Type A from 3 Different Lines of Laboratory Passage.

	Previous laboratory passage		
Time at 61.5° (hr)	Ferret No. 198 593 mice 56 eggs	Ferret No. 198 70 mice 719 tiss. cult. 99 eggs	Ferret No. 198 713 mice 3 eggs
0	20,000+	10,000+	10,000+
¼	10,000+	640	0
½	10,000	160+	0
1	2,560	20+	0
2	640+	0	0
4	80+	0	0

TABLE II.

Effect of Exposure to Temperature of 61.5°C for Varying Intervals on the Hemagglutinin Titers of Allantoic Fluids Containing the Lee Strain of Influenza Virus Type B from 3 Different Lines of Laboratory Passage.

	Previous laboratory passage		
Time at 61.5° (hr)	8 ferrets 137 mice 101 eggs	8 ferrets 338 mice 8 eggs	13 ferrets 0 mice 7 eggs
0	5,000+	10,000+	2,560
¼	5,000+	5,000+	1,280
½	5,000+	5,000+	80+
1	5,000+	2,560	0
2	5,000+	320+	0
4	5,000+	0	0

activity of the PR8 and Lee strains and found in the course of 4 months at 4°C that the Lee strain was less stable than PR8. The observations described in the present report suggest the likelihood that the different findings in different laboratories may be related to the appearance of true variants, with respect to the property in question, that may have developed in the course of passage of the same original strain.

Jones[11] reported the adaptation to heat of the infectious component of influenza virus induced by subjecting the virus-containing suspension to temperatures of 50 to 56°C between successive passages. In personal communication it has been learned that heat resistance was maintained for 3 passages without heat treatment between passages; longer passage without heating has not been studied. It would appear from Jones' studies that the procedure employed resulted in the same effect reported by Armstrong[12] who was able to select a heat-resistant strain of vaccinia virus by passaging samples of virus that had withstood the longest storage periods at 37°C. In Jones' experiments heat-resistant strains did not develop as a result of passage in embryos incubated at temperatures above the range used normally.

Discussion. Evidence for the occurrence of variation in viruses has recently been reviewed by Stanley[13] and by Burnet.[14] Among the plant viruses variants have been shown to possess differences demonstrable chemically and physically as well as biologically.[13] Among the animal viruses the variations that have been described have been in terms of the biological properties of the viruses. For the

[11] Jones, M., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 315.

[12] Armstrong, C., *Pub. Health Rep.*, 1929, 44, 1183.

[13] Stanley, W. M., *Messenger Lectures in Virus Diseases*, Cornell University Press, 1943, 35.

[14] Burnet, F. M., *Virus as Organism*, Harvard University Press, 1945.

influenza viruses it has been shown that different strains of the same type vary in their antigenic makeup[15] as well as in their infective capacities[16] and adaptability for different hosts;[17] strains of virus differ with respect to toxicity[18] and it appears that the capacity for the O to D transformation[19] described by Burnet also varies. Moreover, the characteristics of chicken red cell adsorption-elution, in terms of temperature and time, are different for the PR8 and Lee strains.[1] Studies of certain biophysical properties of a few strains of influenza virus have been made. The biophysical properties which have been considered are related to particle size and electrical charge.[20] With the exception of suggestive variations in size,[21] differences in the properties of strains have not been reported. It would appear reasonable to consider, as another physical property, the resistance to disruption of the biological activities of the virus. From this viewpoint, the present studies have revealed that strains of influenza virus differ widely in at least one biophysical characteristic; namely, the critical temperature of destruction of hemagglutinating activity. It has been found, moreover, that in these terms different lines of the same strain also vary.

In summary it may be said that the absence of uniform reaction in terms of stability to heat, among different strains of virus, and in fact among different lines of the same strain, suggests another approach to the study of the physico-chemical properties of the influenza viruses as well as studies of inheritable variations in these elementary living units.

The practical implications of the present experiments are pertinent to the problem of vaccine antigenicity and stability. The extension of the observations on the stability of the hemagglutinating capacity of the virus to the question of stability of antigenicity is dependent upon the relationship between the hemagglutinating and immunizing properties. Although conclusive evidence of identity, or lack of identity, has not yet been obtained,[2,5] the trend indicates sufficient parallelism to warrant the suggestion that the stability of the hemagglutinin at elevated temperatures may permit predication of stability of immunizing capacity at lower temperatures. Assuming that the same processes occur at the different temperatures, but at different rates, the technic employed in these studies may be of practical value for rapid determination of the manner in which the virus should be manipulated in order to achieve the most stable vaccine. The fact that strains and lines of virus differ in stability suggests the possibility that with strains or lines of similar antigenic valence, the more stable one may be more desirable in a vaccine. While this would appear self-evident, considering the vaccine in the interval between preparation and inoculation, it remains to be seen whether greater resistance to disruption is advantageous after the vaccine has been inoculated. One could speculate either way; *e.g.*, that the more stable virus would be less liable to destruction before it has reached the antibody-producing centers, or that a more stable virus may be less reactive in antibody formation. To what extent these considerations enter the problem of immunization will have to be investigated.

Summary. A report has been made of the observation of variation among different strains and different lines of the same strain of influenza virus with respect to the heat-stability of the hemagglutinating property. Certain theoretical and practical implications of this observation have been discussed.

The technical assistance of Miss Lee Whyte is gratefully acknowledged.

[15] Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

[16] Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.

[17] Francis, T., Jr., *Trans. and Studies of Coll. of Phys. of Phila.*, 1941, **8**, 218.

[18] Henle, W., and Henle, G., *Science*, 1945, **102**, 398.

[19] Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Austral. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 9.

[20] Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80**, 507.

[21] Beard, J. W., Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Feller, A. E., and Dingle, J. H., *Southern Med. J.*, 1944, **37**, 313.

15525

Effect of Formalin in Increasing Heat Stability of Influenza Virus Hemagglutinin.*

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A study of the heat stability of influenza virus hemagglutinin was recently reported[1] in which strain and line variations were described. Additional findings of interest have been revealed in further studies employing the same technics. It is the purpose of this communication to report the observation of an increase in heat-stability of the hemagglutinin of influenza virus in the presence of low concentration of formalin, and to discuss certain extensions of the previous observation of strain differences as evidenced by the variable effect of formalin on several strains of virus.

Materials and Methods. The source of virus in these experiments, as before, was the allantoic fluid of chick embyros infected with the various strains. A constant temperature water bath at 61.5°C was used and the method for titrating hemagglutinin is the same as that employed before. The term formalin refers to a solution containing 40% of formaldehyde by volume.

Results. Formalin, in proper concentration, destroys the infectivity of influenza virus, but the immunizing property is retained.[2] Similarly, formalin will destroy infectivity without altering hemagglutinating capacity.[3] Since an excess of formalin will destroy not only infectivity, but other properties of the virus as well,[3,4] it was anticipated that in the presence of progressively increasing concentrations of the chemical there would occur a corresponding increase in rate of destruction of hemagglutinin at any given temperature. It was of interest to find that with 2 of the 3 strains originally studied, the stability of hemagglutinin at 61.5°C was considerably greater in the presence of 0.05% formalin than in the unformalinized control. Table I illustrates the effect observed when the concentration of formalin was varied in allantoic fluids containing the PR8 and Weiss strains of Type A virus and the Lee strain of Type B virus. From these data it is seen that the stability of the hemagglutinin of the PR8 and Weiss strains was enhanced in the presence of the lowest concentrations of formalin used. In contrast, the Lee strain of Type B virus showed a progressive reduction in stability of its hemagglutinin with increasing concentrations of formalin; even the smallest quantity had a deleterious effect. It is of interest that the 2 Type A strains differed with respect to the degree of difference between the untreated and the formalin-treated preparations and that the range of formalin concentration in which the effect was observed was greater for the Weiss than for the PR8 strain.

In view of these differences a number of other strains was examined to see if a type difference was involved. A group of 13 Type B strains was tested. Among these were the BON strain isolated in Doctor Burnet's laboratory in 1943,[5] 2 strains isolated from an outbreak of May 1945 and 10 strains isolated in this laboratory from the epidemic of 1945-46.[6] Some were tested with different quantities of formalin and others were tested only with the 0.05% concentration. In all 13 an increased stability of hemagglutinin was evident in the presence of the formalin. Table II illustrates the effect of different concentra-

* These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

1 Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134.

2 Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Brit. J. Exp. Path.*, 1935, **16**, 291.

3 Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

4 Eaton, M. D., *J. Immunol.*, 1940, **39**, 43.

5 Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 1.

6 Francis, T., Jr., Salk, J. E., and Brace, W. M., *J. Am. Med. Assn.*, 1946, **131**, 275.

TABLE I.

Rate of Destruction at 61.5°C of Hemagglutinin of 3 Strains of Influenza Virus in Allantoic Fluid Containing Different Concentrations of Formalin.

Strain and passage	Time at 61.5°C (hr)	Concentrations of formalin, %				
		0	0.05	0.10	0.20	0.50
PR8	0	20,000+	20,000+	20,000+	20,000+	20,000+
(Type A)	.5	10,000	20,000+	20,000+	10,000	0
F-198	1	5,000	10,000+	10,000+	2,560	0
M-593	2.5	640+	5,000+	2,560	0	0
E-56	6	0	640+	0	0	0
	8	0	80+	0	0	0
WEISS	0	10,000+	10,000+	10,000+	10,000+	10,000+
(Type A)	.25	5,000+	10,000	5,000+	5,000+	2,560
F-3	.5	1,280+	5,000+	5,000+	5,000+	40
M-32	1	160+	5,000+	5,000+	5,000+	0
E-54	2	0	5,000+	5,000+	2,560+	0
	4	0	1,280	2,560+	640	0
	8	0	640	640	80	0
LEE	0	10,000+	10,000+	10,000+	10,000+	10,000+
(Type B)	1.5	10,000	10,000	160+	0	0
F-8	3	10,000	1,280	0	0	0
M-137	6	5,000+	0	0	0	0
E-102	9	5,000+	0	0	0	0
	16	1,280	0	0	0	0

TABLE II.

Rate of Destruction at 61.5°C of Hemagglutinin of the Amdall Strain* of Type B Influenza Virus in Allantoic Fluid Containing Different Concentrations of Formalin.

Time at 61.5°C (hr)	Concentrations of formalin, %						
	0	0.01	0.025	0.05	0.10	0.25	0.50
0	5000	5000	5000	5000	5000	5000	5000
.25	0	640+	2560	5000	5000	1280+	0
.5	0	320	1280+	2560+	5000	160	0
1	0	80	640	1280+	1280+	0	0
2	0	0	320	1280	1280	0	0

* In sixth egg-passage.

tions of formalin on one of the recently isolated Type B strains and Table III shows the effect of 0.05% formalin on several other strains as well. In terms of the effect of formalin in a concentration of 0.05%, it is seen that variations exist among the strains shown in Table III, all of which had been isolated from the same outbreak. It appears from these data that the Lee strain is exceptional among the Type B strains, thus far tested, with respect to the effect of the presence of formalin on the stability of the hemagglutinating component of the virus. In view of the variation in heat-stability of 3 different passage lines of the Lee strain reported previously,[1] it was of interest to find that all 3 lines behaved in the same exceptional way in the presence of formalin.

Table IV illustrates the observations made in tests of 3 Type A strains isolated in this laboratory, other than PR8 and Weiss. All 3 showed a slight enhancement of stability in the presence of formalin, but the effect was strikingly less marked than in the case of the Type B strains shown in Tables II and III. Moreover, a somewhat higher concentration of formalin was required to produce the slight effect evident in the Type A strains shown in Table IV.

In summary, these data show that in the

TABLE III.

Rate of Destruction of Hemagglutinin of 5 Strains of Type B Influenza Virus from the 1945-46 Epidemic. Comparison of Formalinized and Unformalinized Allantoic Fluids.

Time at 61.5°C (hr)	Amdall		Neubert		Sadowski		Mindell		Baker	
	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.	No Formal.	.05% Formal.
0	2560+	2560+	2560+	2560+	2560+	2560+	1280+	1280+	1280+	1280+
.25	0	—	0	—	0	—	0	—	*0	*160+
1	0	2560+	0	1280	0	640+	0	1280	*0	*40
2	0	2560	0	640	0	320	0	640+	*0	*0
4	0	1280	0	320	0	20+	0	320		
6	0	320	0	80	0	0	0	80		
8	0	20+	0	0	0	0	0	0		
10	0	0	0	0	0	0	0	0		

* Successive intervals were 5, 10, 15 minutes instead of time shown in first column.

TABLE IV.

Rate of Destruction at 61.5°C of Hemagglutinin of 3 Strains of Type A Influenza Virus in Allantoic Fluids Containing Different Concentrations of Formalin.

Strain and passage	Time at 61.5°C (min)	Concentrations of formalin, %						
		0	.01	.025	.05	.10	.25	.50
Johnson (1943) Type A F=0 M=0 E=9	0	5000+	5000+	5000+	5000+	5000+	5000+	5000+
	15	20	20	40+	160+	160+	640+	40+
	30	0	0	0	20+	160	320	0
	60	0	0	0	0	20+	20+	0
	120	0	0	0	0	0	0	0
Scott (1943) F=0 M=0 E=7	0	2560+	2560+	2560+	2560+	2560+	2560+	2560+
	5	0	0	0	20	40+	0	0
	15	0	0	0	0	0	0	0
Baum (1941) Type A F=1 M=10 E=11	0	10,000	10,000	10,000	10,000	10,000	5000+	0
	5	640+	640+	2,560	2,560+	2,560+	0	0
	15	0	0	0	40+	320+	0	0
	30	0	0	0	0	0	0	0

presence of small concentrations of formalin the hemagglutinin of all but one of 19 strains of influenza virus tested is more stable when subjected to a temperature of 61.5°C and that the effect on different strains varies.

In an effort to determine the influence of time on the reaction between formalin and the hemagglutinating factor tests were made of heat stability at intervals after storage at 4°C. In mixtures of formalin and the Weiss or PR8 strains tested immediately after preparation or after storage for 2 days or 2 weeks, the inactivation curves were indistinguishable, provided the concentrations of formalin present were not great enough to cause destruction of hemagglutinin at the 4° storage temperature. In another experiment, in which formalin was added *after* the virus preparation had been heated for different periods of time, the hemagglutinating capacity was not restored; although in an aliquot the hemagglutinating capacity was retained when formalin had been added *prior* to heating for corresponding intervals. It would seem, therefore, that the presence of formalin delays the destructive effect of heat on hemagglutinin; however, the addition of formalin to a heated virus suspension does not reactivate the hemagglutinin.

Discussion. The observations reported in this communication are of interest with respect to several questions.

It has been noted with almost all strains of influenza virus examined that the heat-stability of the hemagglutinating property was enhanced in the presence of low concentrations of formalin. This is similar to the finding of Schmidt, Glenny, Ramon, and others who reported that the stability of diphtheria toxin to heat-denaturation was increased after treatment with formaldehyde for production of toxoid.[7] It is also known that formalinized pneumococci are more stable than unformalinized organisms[8] and that greater stability to the denaturing action of heat can be produced by formaldehyde treatment of proteins.[9] The exact mechanism of the formaldehyde effect, in which toxicity or infectivity is destroyed without impairing other antigenic properties, is unknown. Many hypotheses have been suggested.[7,9,10] The present observations support the speculation that in addition to blocking or destroying the chemical groupings necessary for toxicity or infectivity, some intramolecular rearrangement occurs producing an effect that might be described as "fixation" of other reactive groups.

A curious fact with respect to influenza virus is the variation observed in the behavior of the hemagglutinating factor of different strains in the presence of formalin. Lack of uniformity in the reaction of the various strains of virus with so simple a substance as formaldehyde suggests that the chemical groupings in the constitution of the individual strains differ widely, either quantitatively or qualitatively.

It is of further interest that differences in the formalin effect were observed among strains of virus isolated from different patients in the same geographical area during a recent epidemic of influenza B.[6] These same strains have been found to possess differences in serological[6] and physical[1] properties. Variations in virulence and serological differences among strains of influenza virus have been described previously[11] and the problems posed by strain variations have been discussed by Francis,[12] Andrewes,[13] Burnet,[14] and others. The fact that strains of virus from the same outbreak are intrinsically different indicates that variants manifested by differences in certain properties do occur very readily in nature. An agent, so labile in its genetic constitution as to vary in the course of multiplication in the same host or from host to host, may be expected to alter its biological behavior quite unpredictably. This characteristic may serve to explain, in part, the striking variability of influenza virus activity both clinically and epidemiologically.[15]

With respect to the more immediately practical question of influenza virus vaccines, the finding of an increased stability of the virus hemagglutinin after formalinization of all but one of the strains tested suggests that the problem of a useful inactivating agent had been solved. The exact relationship between hemagglutinating activity and immunizing activity is still under investigation. The trend of studies reported[16,17] as well as those in progress invites the speculation that the technics employed may furnish information of value with respect to the relative stability of the immunizing capacity of influenza virus vaccines prepared or treated in various ways.

Summary. The observation of an increase in heat-stability of the hemagglutinating property of influenza virus in the presence of low concentrations of formalin has been described. Differences in the effect of formalin on different strains has been reported and discussed.

The technical assistance of Miss Lee Whyte is gratefully acknowledged.

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[8] Tao, S. M., *Chinese Med. J.*, 1932, **46**, 12.

[9] Levaditi, C., Lepine, P., and Verge, J., in *Les Ultravirus des Maladies Animales*, Maloine, Paris, 1943, pp. 108-110.

[10] Boyd, W. C., in *Fundamentals of Immunology*, Interscience Publisher, New York, 1943, pp. 104-105.

[11] Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.

[12] Francis, T., Jr., *The Harvey Lectures*, 1941-42, Series 37, 69.

[13] Andrewes, C. H., *Proc. Roy. Soc. Med.*, 1942, **36**, 1.

[14] Burnet, F. M., in *Virus as Organism*, Harvard Univ. Press, 1945, p. 109.

[15] Francis, T., Jr., *J. Am. Med. Assn.*, 1943, **122**, 4.

[16] Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1.

[17] Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

15526 P

Bilirubin, Bromsulfalein, Bile Acids, Alkaline Phosphatase and Cholesterol of Thoracic Duct Lymph in Experimental Regurgitation Jaundice.

MIGUEL V. GONZALEZ-ODDONE. (Introduced by Cecil J. Watson.)

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Saunders[1] was the first to ligate the hepatic duct in dogs in 1795. Since then many others have attempted to elucidate the problem of regurgitation jaundice. An outstanding contribution and review on the subject is given by Mayo and Greene.[2]

The purpose of the present study was to determine the content of bilirubin, bile acids, alkaline phosphatase, and cholesterol, and of bromsulfalein following its administration intravenously, in the plasma and thoracic duct lymph of dogs, after ligation of the common bile duct.

Methods and Materials. Five male dogs were used ranging from 10 to 15 kg. Thirty to 40 mg of pentobarbital sodium per kg of body weight were used as anesthesia. The thoracic duct was cannulated at its entrance in the left jugular vein. Three drops of *25%* sodium citrate solution were added to 10 cc of blood or lymph, to prevent coagulation, all determinations being made on plasma. Biliary obstruction was produced by a double ligation and severance of the common bile duct with previous ligation of the cystic duct. Bilirubin was determined by a modification[3,4] of Malloy and Evelyn's method.[5] Bromsulfalein was administered in the right external jugular vein in a dose of *2* mg per kg of body weight. The concentration of dye was then determined in the following manner:

Three cc of triethyl phosphate (Eastman Kodak Company) was added to 1 cc of plasma or lymph in a centrifuge tube. This was shaken thoroughly and centrifuged. Two cc of the supernatant fluid were then added to 8 cc of distilled water in an Evelyn tube. A blank reading was made in the Evelyn photoelectric colorimeter, using a 580 filter. Two drops of *20%* KOH were then added to develop the color, and a reading was again taken, after which the concentration was de-

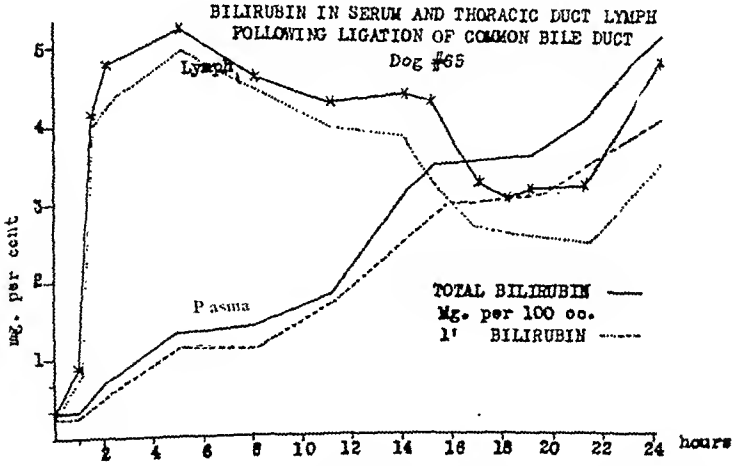

FIG. 1.

1 Saunders, W. A., *A Treatise on the Structure, Economy, and Disease of the Liver*, William Phillips, London, 1809.

2 Mayo, C., and Greene, C. H., *Am. J. Physiol.*, 1929, **89**, 280.

3 Ducci, H., and Watson, C. J., *J. Lab. and Clin. Med.*, 1945, **30**, 293.

4 Watson, C. J., *Blood*, 1946, **1**, 99.

5 Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.*, 1937, **119**, 480.

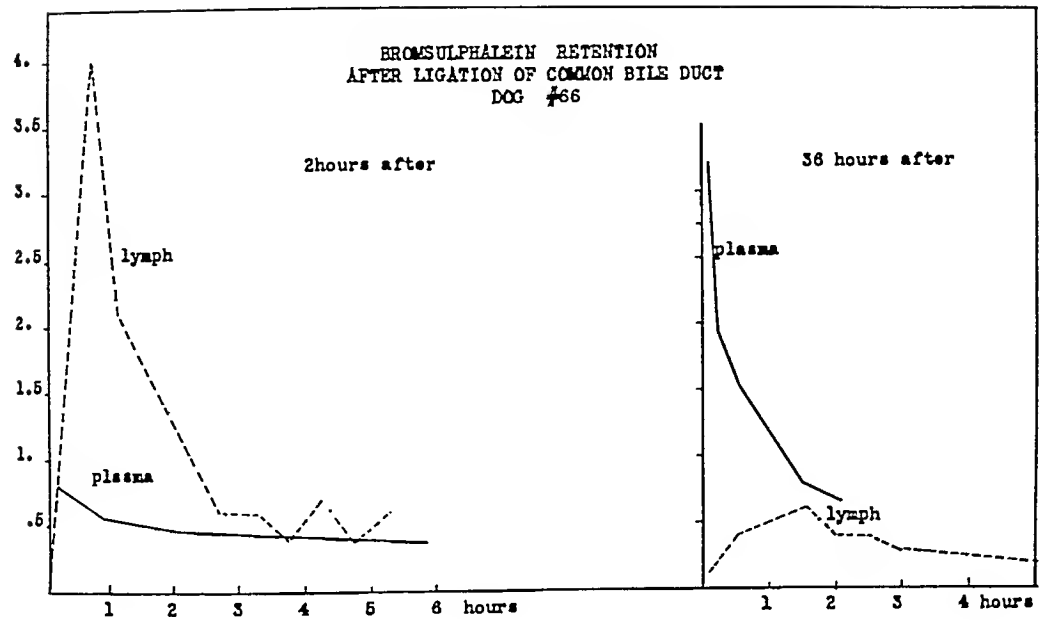

FIG. 2.

termined from a curve of the pure dye in known concentration. Bile acids were determined as cholic acid by Josephson's method.[6] Cholesterol was determined by the Sperry Schoenheimer method.[7] The alkaline phosphatase was determined by the method of King and Armstrong.[8]

Results. As seen in Fig. 1, bilirubin appears promptly in the thoracic duct lymph, being detectable in the blood a few hours later. Most of the bilirubin was of 1′ or prompt reacting type[3,4] in both lymph and plasma.

The data in Fig. 2 relates to injection of bromsulfalein at 2 hours and at 36 hours following the ligation of the common bile duct. In the early period of the obstruction, it is seen that the dye disappears promptly from the blood to be regurgitated at high concentration into the thoracic duct lymph. In the later period of obstruction, however, the dye is retained in the blood for several hours and little or none appears in the thoracic duct lymph.

[6] Josephson, B., *Biochem. J.*, 1935, **29**, 1519.

[7] Schoenheimer, R., and Sperry, W. H., *J. Biol. Chem.*, 1934, **106**, 745.

[8] King, E. J., and Armstrong, A. R., *Canad. Med. Assn. J.*, 1934, **31**, 376.

Fig. 3 represents a typical experiment as regards bile acids in the thoracic duct lymph and blood, following ligation of the common bile duct.

These results are in accord with previous studies in which the Pettenkofer test was used, as discussed by Mayo and Greene.[2]

Typical data for the cholesterol in lymph and blood, in these experiments, is shown in Fig. 4. It is seen that the concentration of the total plasma cholesterol remained about the same during the 24 hours of observation. From this, it appears that if regurgitation of cholesterol from bile to lymph and thence to blood, does occur, it is considerably slower than that of the bile acids and the prompt reacting bilirubin.

Fig. 5 shows the rise in alkaline phosphatase in the blood and lymph. It is of considerable interest that distinct increases in phosphatase activity in thoracic duct lymph were observed.

Summary. The present results support the belief that, following common duct ligation, bile "regurgitates" into the lymph, and thence, via the thoracic duct into the blood. The appearance of bile acids and bilirubin in the thoracic duct lymph, as previously reported, is confirmed. The present study also reveals

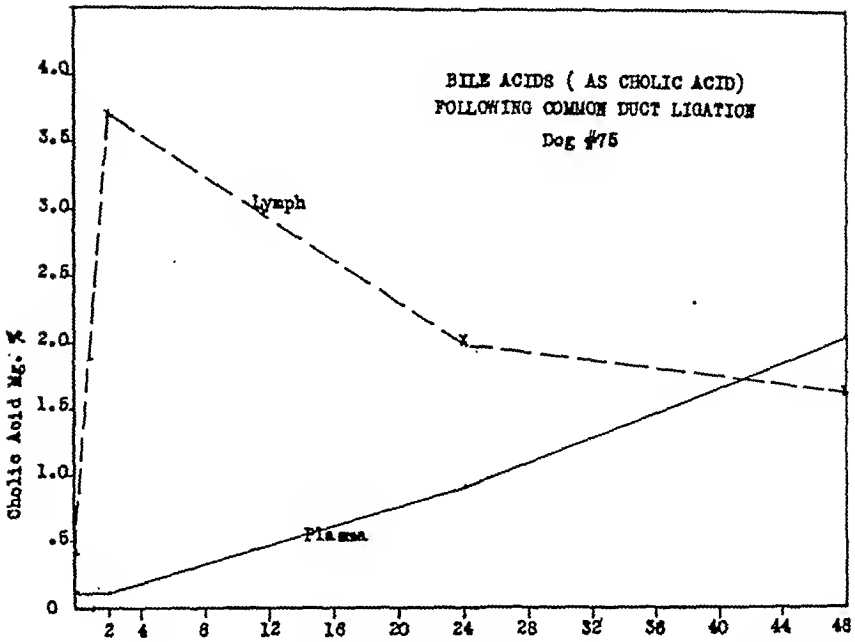

FIG. 3.

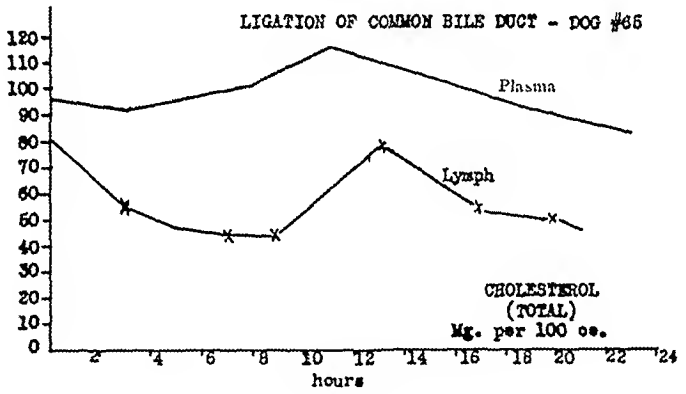

FIG. 4.

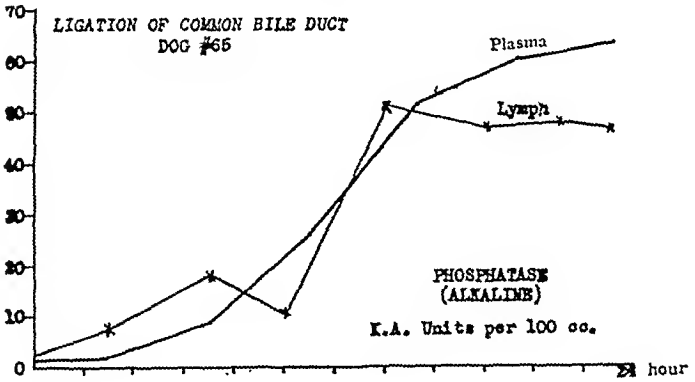

FIG. 5.

that the bilirubin of the lymph, under these circumstances, is mainly of the prompt reacting type, a finding in accord with the concept of a regurgitation of bile into the lymph.

During the early phase of biliary obstruction, the injected bromsulfalein quickly appeared in the lymph. After 36 hours, however, it was not removed from the blood and did not appear in the lymph in appreciable amount.

The cholesterol, on the contrary, did not increase significantly in the lymph within 24 hours, while the alkaline phosphatase behaved in an intermediary manner.

The writer wishes to acknowledge his indebtedness to Dr. C. J. Watson for helpful criticism and advice with relation to these studies.

15527

Effect of Anoxic Anoxia on Gastric Emptying Time of Rats Fed Corn Oil.

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Anoxic anoxia has been shown to cause a delay in the emptying time of the stomach in the dog and in man. Moreover, in man, as in the case of the dog, the more severe the degree of anoxia, the greater is the delay in gastric emptying.[1]

Studies on the effect of this type of anoxia on the absorption of fat from the alimentary tract in rats have shown that the amount of fat absorbed by animals subjected to partial pressures of oxygen of 63 mm and 53 mm Hg was significantly less than for control animals; the difference was greater for the greater degree of anoxia.[2] That these findings cannot be explained on the basis of a prolonged emptying time of the stomach, however, is shown by the following experimental results.

Using the technic previously described,[2] adult albino rats of both sexes, weighing between 200-300 g were given 1.5 cc corn oil (1.385 ± 0.01 g) and subjected to a partial pressure of oxygen of 53 mm Hg (7.03% oxygen) for 2-, 3- and 4-hour periods. Simultaneously-fed control animals were kept at atmospheric pressure. The amount of fat remaining in the stomach was quantitatively determined and calculated as per cent of the amount fed. Preliminary experiments on rats killed immediately after feeding showed that the mean percentage recovery of the amount of fat administered was 96.5 (range 92.3 to 99).

The data, Table I, show that the amount of

[1] Van Liere, E. J., *Physiol. Rev.*, 1941, **21**, 307.

[2] MacLachlan, P. L., and Thacker, C. W., *Am. J. Physiol.*, 1945, **143**, 391.

TABLE I.
Effect of Anoxic Anoxia on Gastric Emptying Time of Rats Fed Corn Oil.
Oxygen tension—53 mm Hg (7.03% oxygen).

Absorption time	2 hr		3 hr		4 hr	
	No. of rats	Fat in stomach* %	No. of rats	Fat in stomach %	No. of rats	Fat in stomach %
Controls	12	68.4	11	49.5	12	41.3
Anoxic	11	37.4	12	30.0	12	35.7
St. Dev.		17.0		16.5		13.4
P. (Fisher's)		<0.01		0.01		>0.3

* Expressed as per cent of the amount of fat fed.

fat remaining in the stomachs of the anoxic rats 2 hours and 3 hours after feeding was significantly less than for the corresponding controls. On the other hand, no statistically significant difference was found at the end of 4 hours. (The individual variation which was greater for the rats subjected to anoxia, than for the controls, accounts for the apparently larger amount of fat in the stomach after 4 hours absorption time than after 3 hours). Although a delay in gastric emptying may have occurred later than 4 hours, these findings indicate an initial acceleration of the emptying of the stomach of rats exposed to diminished oxygen tension. For the purpose of these experiments, the results obtained show that the decreased rate of absorption of fat in rats subjected to anoxic anoxia, as previously reported, cannot be explained on the basis of a prolonged gastric emptying time.

Schnedorf and Orr[3] reported that increasing degrees of anoxemia produced by inhalation of 15, 10 and 5% oxygen in nitrogen resulted in a marked and progressive decrease below normal in the flow of bile in nembutalized dogs. The decreased rate of fat absorption observed in rats subjected to partial pressures of oxygen of 63 mm and 53 mm Hg (8.35 and 7.03% oxygen, respectively) might reasonably result from a diminished flow of bile. Work on this phase of the problem is in progress.

Summary. Adult albino rats fed corn oil showed an initial acceleration of the emptying of the stomach on exposure to diminished oxygen tension. A decreased rate of absorption of fat in rats subjected to anoxic anoxia cannot be explained on the basis of a prolonged gastric emptying time.

[3] Schnedorf, J. G., and Orr, T. G., *Am. J. Dig. Dis.*, 1941, **8**, 356.

15528

Latent Period Between Electrical and Pressure Pulse Waves Corresponding to Right Auricular Systole.*

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The exact time relationship between the onset of electrical activity and the appearance of a pressure wave corresponding to auricular systole has been, heretofore, unknown in man. The development of the technic of catheterization of the right heart[1-4] and the use of a multiple blood pressure recorder with a synchronous electrocardiogram has made possible the study of this time relationship.

Preliminary tests for measuring the difference of transmission time of electrical impulses as recorded with the ECG and simultaneous mechanical impulses from the tip of a catheter connected with a Hamilton manome-

* Under grants from the Commonwealth Fund and the Life Insurance Medical Research Fund Gift for Study of Action of Certain Cardiovascular Drugs.

† Captain Medical Corps Reserve, U. S. Army, on detached service from Aero-Medical Laboratory, Wright Field, Dayton, Ohio.

[1] Cournand, A., and Ranges, H. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 462.

[2] Cournand, A., Lauson, H. D., Bloomfield, R. A., Breed, E. S., and Baldwin, E. deF., PROC. SOC. EXP. BIOL. AND MED., 1941, **55**, 34.

[3] Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr., *J. Clin. Invest.*, 1945, **24**, 106.

[4] Cournand, A., Bloomfield, R. A., and Lauson, H. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 73.

TABLE I. Time Relationship of Electrical and Pressure Pulse Waves Corresponding to Auricular Systole in Man.

Case	Date	Clinical diagnosis	Age, yrs	Heart rate	Avg. time in sec.*			
					Beginning of P wave to beginning of auricular systole	Peak of P wave to peak of auricular systole	Beginning of P wave to peak of auricular systole	P-R interval of ECG
			8 Cases with Essentially Normal Cardiac Function.					
L.H.	1-29-36	Pulmonary fibrosis	50	73.2	—	.12	.16	.16
B.O.	2-19	Essentially normal	27	78.2	—	.095	.15	.15
E.H.	2-25	"	23	85.2	.11	.11	.16	.16
E.H.	3-12	"	23	85.6	.11	.11	.16	.16
E.L.	3-5	Art. pneumothorax	27	90.8	.095	.095	.17	.19
J.L.	3-26	6 yrs post pneumonectomy	18	72.0	.13	.12	.18	.17
E.C.	4-15	Essentially normal	31	81.2	.11	.11	.14	.17
A.S.	4-15	Art. pneumothorax	22	111.5	—	.13	.16	.15
	Avg.			86.0	.11	.11	.160	.164
			2 Cases with Cardiac Abnormalities.					
G.L.	3-19	Pathology of Pallor	8	113.0	.055	.055	.125	.13
E.H.	3-28	Scleroderma	50	83.0	.17	.17	.20	.25

* Corrected for .01 sec difference between electrical and mechanical conduction time.

Fig. 1.
A. Mechanical conduction.
B. Electrical conduction.
Transmission time difference of simultaneously-induced electrical and mechanical impulses. (See text for description).

ter were made in the following manner: one electrode of the ECG was attached to a small rubber balloon, fastened on the distal end of a No. 8 cardiac catheter, with the proximal end connected by lead tubing to a Hamilton manometer. The entire mechanical system was filled with sodium citrate with all air bubbles removed. Tapping the attached electrode with another electrode (circuit of lead II used), initiated simultaneously an electrical and mechanical impulse that was transmitted to the string of the ECG and the manometer membrane; the resulting deflections were photographed using a camera speed of 50 mm per second. As illustrated on Fig. 1, the time interval between the beginning of the electrical and the mechanical deflection is approximately 0.01 second.

The data obtained on 10 human subjects are tabulated in Table I, and representative tracings shown on Fig. 2. In 8 individuals with essentially normal cardiac function the latent period of the right auricle measured from the time of the beginning of the P wave to the beginning of the auricular systolic pulse wave averaged 0.11 second after the 0.01

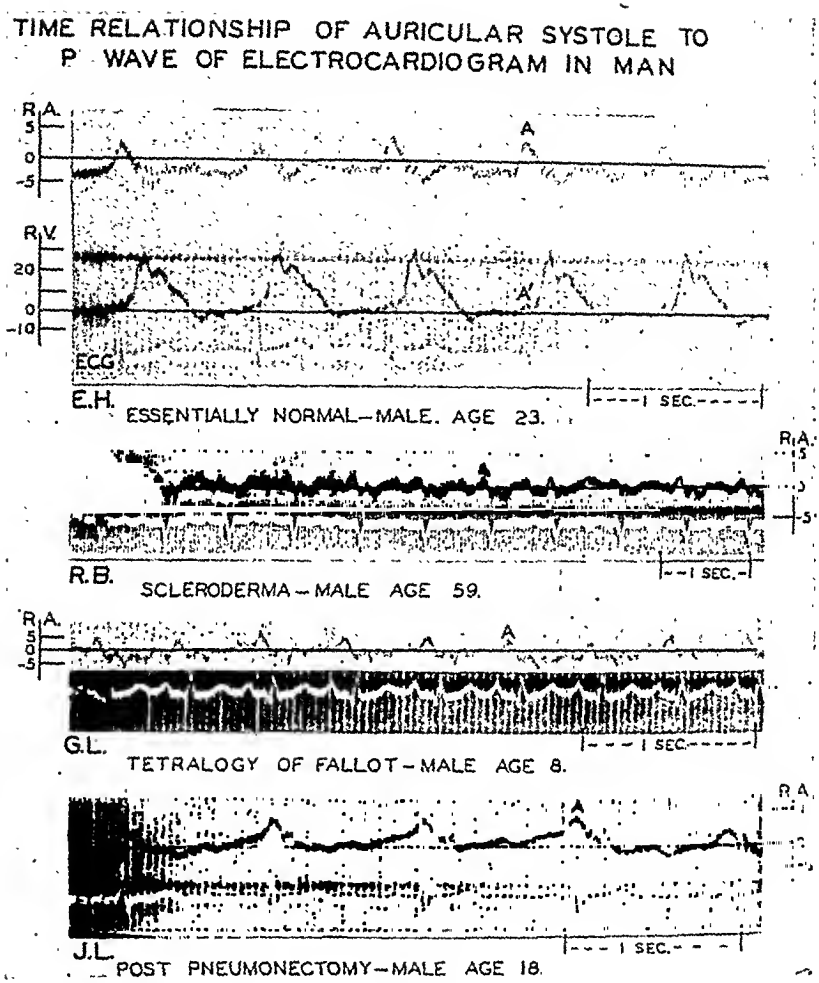

Fig. 2.

A. = auricular systole.
A'. = auricular systole on right ventricle tracing.
R.A. = right auricle. R.V. = right ventricle.
E.C.G. = electrocardiogram—lead II.
All pressures in mm mercury.
Time interval between electrical and pressure pulse wave corresponding to the auricular systole in normal man and in 2 cases with cardiac abnormalities.

second correction was subtracted for the difference between the electrical and mechanical conduction. In 3 cases it was necessary to take the measurements from the peak of both electrical and pressure waves as the exact beginning of the auricular systole was not sharply defined on the tracing. In all cases where both determinations could be made the time lag was the same as seen in Table I. The average time from the beginning of the P wave to the peak of the auricular systolic pulse wave was 0.16 second, ranging from 0.14 sec. to 0.18 sec. The P-R interval averaged 0.164 sec. (range 0.15 sec. to 0.19 sec.)

The results in 2 cases of cardiac abnormality are shown in Table I and the tracings illustrated in Fig. 2. In the case of an 8-year-old child with Tetralogy of Fallot, the time lag between the electrical and pulse wave was 0.055 sec. The heart rate was rapid, 113 per minute. The time from the beginning

of the P wave to the peak of the auricular systole was shorter than the normal limits calculated above. In one case of a 59-year-old male diagnosed as scleroderma with involvement of the lungs, marked pulmonary hypertension and evidence of partial heart block, the latent period was 0.17 sec. The time from the beginning of the P wave to the peak of the auricular systole was 0.20 sec. in this case. This may represent some delay in the spread of the sinus impulse through the right auricle.

Summary. 1. In 8 adult subjects with essentially normal hearts the latent period between the beginning of the electrical P wave and pressure pulse wave corresponding to the right auricular systole averaged 0.11 sec. 2. In one case of a child with a rapid pulse and Tetralogy of Fallot the latent period was much shorter, 0.055 sec. 3. In one case of scleroderma with pulmonary hypertension and partial heart block the latent period was prolonged to 0.17 sec.

15529 P

Influence of Thiourea on Development of the Chick Embryo.

N. GROSSOWICZ.* (Introduced by I. Olitzki.)

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While investigating the effect of several substances which may influence the growth of rickettsiae in the yolk sac of the chick embryo, some effects on the embryo itself have been observed, and these have been followed up for thiourea.

Thiourea and related substances have been shown to affect the thyroid function in adult animals, *viz.*, rat,[1,2] mouse and dog,[1] rabbit,[3] and chick.[4] Thiourea has also been found to inhibit metamorphosis in tadpoles[5-7] and cleavage of sea urchin eggs.[8]

Thiourea is introduced into the yolk sac of Leghorn embryos according to the technic described by Cox.[9] Aseptic precautions are strictly adhered to throughout the whole procedure. Fertile eggs incubated at 39°C for 7-17 days are used. The shell covering the air sac is washed with 5% phenol and the injections are made into the yolk through a needle-sized opening in the air sac by means of a hypodermic syringe and a 22-gauge needle. The hole is then sealed with paraffin and the eggs are returned to the incubator. Amounts of thiourea used were 0.3 to 3 mg per egg. In order to avoid mechanical damage to the embryo, the fluid introduced should not exceed 0.5 ml; a similar volume of distilled water serves as control. Eggs were daily inspected by candling and dead ones were discarded.

Results. While the controls hatched after 21 days (on very few occasions after 20 or 22 days), hatching of embryos treated with thiourea was retarded up to 10 additional days. The retardation seemed to depend on 2 factors, (a) age of the embryo at the time of injection; (b) concentration of thiourea. For example, 1 mg thiourea delayed hatching by only 1 day in 17-day egg embryos, while the same amount applied to younger

* The author is indebted to Dr. M. Aschner for his valuable suggestions.

1 Mackenzie, C. G., and Mackenzie, I. B., *Endocrin.*, 1943, **32**, 185.

2 Astwood, E. B., Sullivan, J., Bissel, A., and Tyslovitz, R., *Endocrin.*, 1943, **32**, 210.

3 Bauman, I., Metzger, N., and Marine, D., *Endocrin.*, 1944, **34**, 44.

4 Mixner, J. P., Reineke, E. P., and Turner, C. W., *Endocrin.*, 1944, **34**, 169.

5 Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Nature*, 1943, **152**, 504.

6 Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Growth*, 1945, **9**, 19.

7 Hughes, A. M., and Astwood, E. B., *Endocrin.*, 1944, **34**, 138.

8 Bevelander, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 268.

9 Cox, H. R., *Science*, 1941, **94**, 399.

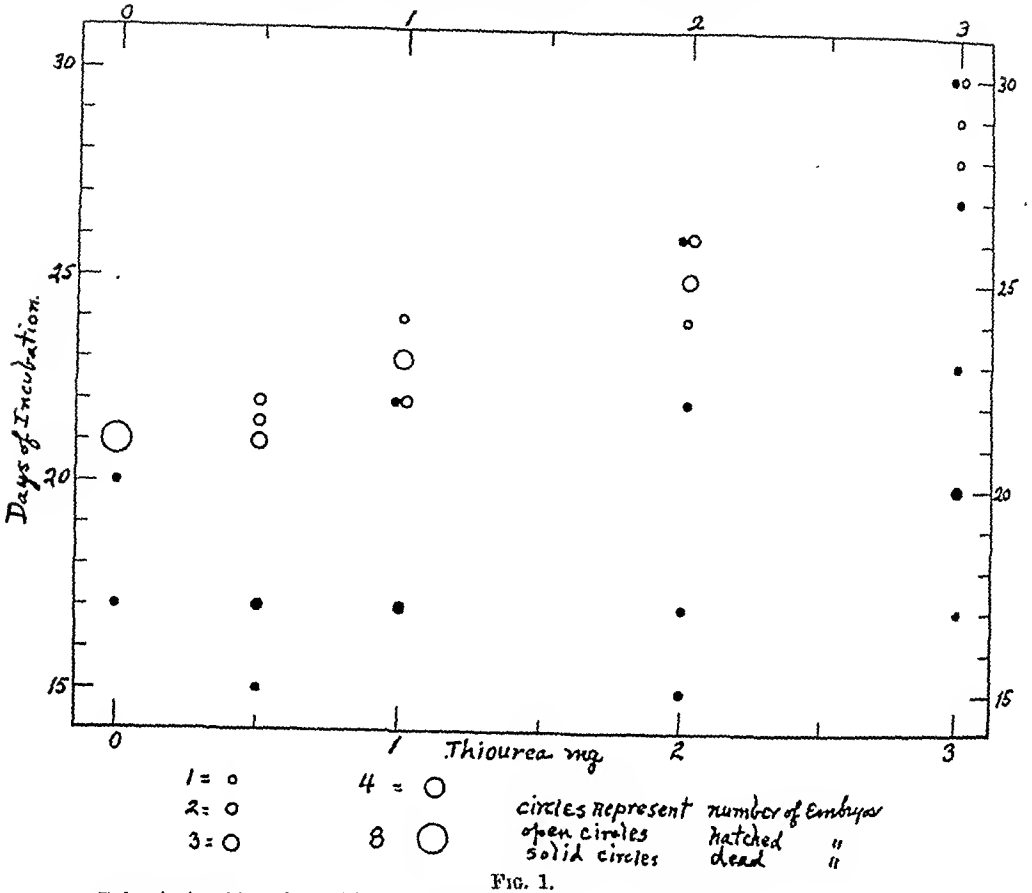


FIG. 1.
Delay in hatching time of Leghorn egg embryos after injection of thiourea at the 14th day of incubation.

embryos (10 days old) caused a delay of 3 days. Two mg of thiourea retarded hatching by 3-7 days, the effect again depending on age. Though hatching time can be extended up to the 30th day by still higher amounts of thiourea (3 mg), hatchability is much impaired being equivalent to only about 5-10% of the controls. In addition, the delay in hatching is associated with other signs of inhibition. Whereas in normal embryos the yolk sac begins to retract on the 19th day, and by the 20th day has entirely entered the abdomen, the yolk sac is not much reduced in size at this stage in eggs treated with thiourea; it can still be found on the 30th day of incubation in those embryos previously treated with a dose of 3 mg thiourea. While in the controls the "egg tooth" enters the air-chamber on the 20th day, treatment with 2 mg thiourea at age of 10-14 days delays this phenomenon by 2-5 days. The results of a typical experiment are given in Fig. 1.

Experiments were also carried out in order to elucidate the mode of action of thiourea. Gordon *et al.*[6] were able to show that the inhibiting effect of thiourea on tadpoles can be reversed by thyroxine. In our experiments it was difficult to prove the reversing effect of thyroxine as it was found to be very toxic especially for embryos up to 14 days. Thyroxine in nontoxic concentrations had no antagonizing activity at all. However, in some experiments with 17-day-old eggs, 10 μg of thyroxine fully neutralized the action of 2 mg thiourea when the 2 drugs were simultaneously applied (molecular ratio 1:2,000). The experiments suggest that in the chick embryo, as in other animals, the inhibiting effect of thiourea may be due to interference with normal thyroxine metabolism.

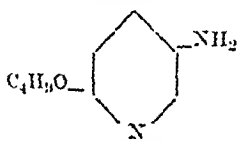
15530 P

A New Class of Tuberculostatic Substances.

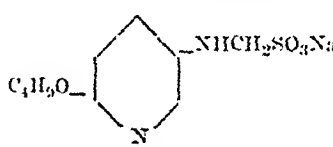
W. Harry Feinstone.*

From the Research Laboratories, Pyridium Corporation, Yonkers, N.Y.

The search for substances having tuberculostatic activity has in recent years engaged increasing interest in many laboratories. Using an *in vitro* technic in which a rapidly growing avirulent strain of "human" tuberculosis organism is subjected to various concentrations of compounds under test makes feasible the "screening" of large numbers of substances in a comparatively short time. This method, the details of which are essentially the same as described earlier,[1] has brought to attention 5-amino-2-butoxypyridine



which was investigated in some detail. Several hundred derivatives of this substance were synthesized[†] one of which, the sodium formaldehyde bisulfite derivative



relatively among the least toxic,[2] is included in this report.

The growth of 607, the rapidly growing strain of the tubercle bacillus, was inhibited by these compounds in dilutions in excess of 1 to 3 million. Other strains, virulent and recent isolations, were inhibited in dilutions as high as 1 to 100 million. A remarkable feature associated with this high tuberculostatic effect is that while several bacterial genera have been investigated only Mycobacterium was susceptible to the action of these substances in significant dilution. The data presented in Table I are typical of the results obtained in many-time repeated experiments. The specificity for Mycobacterium is illustrated by the lack of inhibition of growth of Staphylococcus, Streptococcus, Pneumococcus, *B. mycoides, E. coli,* and others, even in what may be termed high concentrations. This specificity contrasts sharply with the lack of specificity shown by sulfones and sulfonamides[3] which are active against the rapidly growing avirulent *Mycobacterium tuberculosis 607* (line No. 1) as well as *E. coli* in a synthetic medium (line No. 10). Comparison is also made with promin and its parent substance p,p'-diaminodiphenylsulfone.

It is important also to note that while the more active sulfonamide and sulfones are capable of inhibiting the growth of the avirulent strain of tubercle bacillus in comparatively high dilution (line No. 1), these compounds are relatively ineffective against other more virulent strains (lines 2, 3 and 4).

The antagonistic effect of para-aminobenzoic acid[3] and methionine[4] for sulfonamides is well known. As suspected these substances did not inhibit the activity of I or II, as shown by lines 1, 14 and 15 in Table I. The bacteriostatic activity of our compounds against the tubercle bacillus is likewise not antagonized by the incorporation of adequate quantities of riboflavin, calcium pantothenate, adenine, guanine, thiamine, uracil, nicotinic acid, biotin, culture filtrates from staphylococci, pneumococci and tubercle bacilli, pus from streptococcal lymphadenitis, constituents from beef culture media, peptone (which

* The author wishes to express his grateful acknowledgment of the technical assistance rendered by Miss Anna Kelly and Miss Mary Rothlauf.

[1] Fitzgerald, R. J., and Feinstone, W. H., Proc. Soc. Exp. Biol. and Med., 1943, 52, 27.

[†] 5-Amino-2-butoxypyridine and its derivative were synthesized by the chemical staff of the Pyridium Corp. under the supervision of Drs. E. T. Tisza and H. L. Friedman.

[2] To be reported elsewhere.

[3] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[4] Harris, J. S., and Kohn, H. I., *J. Pharm. and Exp. Therap.*, 1942, **73**, 383.

TABLE I.

Comparative Bacteriostatic Effect of 5-Amino-2-Butoxypyridine and Certain Sulfonamides and Sulfones.

	Organism	Culture medium	Lowest concentration (mg per 100 cc) showing bacteriostasis. Compounds					
			II†	II§	Sulfanilamide	Sulfathiazole	Promin§	p,p'-Diaminodiphenylsulfone‡
1	*M. tbc.* 607‖	Dorset's Synthetic	0.031	0.062	2.0	0.062	4.0	0.062
2	*M. tbc.* H37RV	Proskauer Beck	0.0039	0.0078		0.5	64.0	0.5
3	*M. tbc.* G2¶	,, ,,	0.00099	0.00099	64.0	2.0	16.0	2.0
4	*M. tbc.* Benton**	,, ,,	0.0019	0.00099	32.0	0.5	16.0	2.0
5	*M. tbc.* bovine‖	,, ,,	0.0078	0.015				
6	*M. avium*‖	,, ,,	0.156	0.0312				
7	*M. phlei*‖	,, ,,	0.0039	0.0039				
8	*M. stercoris*‖	,, ,,	0.0039	0.015				
9	*M. lepræ*‖	,, ,,	0.031	0.031				
10	*E. coli*	*Sayhun Synthetic*	*64.0*	*64.0*	2.0	0.031	10.0	1.0
11	*Strep. hem.* C203	Beef Infusion Broth	32.0	64.0	32.0	16.0		10.0
12	*B. mycoides*	Peptone Dextrose Broth	8.0	32.0		8.0		
13	Pneumococcus SV1	,, ,, ,,	64.0	64.0	8.0	0.5		
14	*M. tbc.* 607	Dorset's + 0.1 mg % para-aminobenzoic acid	0.031	0.062	64.0	8.0		8.0
15	*M. tbc.* 607	Dorset's + 1.0 mg % methionine	0.031	0.062	16.0	0.25		1.0

* Complete or almost complete visible inhibition of growth for 72 hours following inoculum or in the case of slow growing tubercle bacillus for 30 days. Tubercle bacilli inoculum either by small pellicle or suspension of about 20,000 organisms per cc.

† I—5-Amino-2-butoxypyridine as the hydrochloride.

‡ II—Sodium formaldehyde bisulfite of 5-amino-2-butoxypyridine.

§ Promine and p,p'-diaminodiphenylsulfone supplied through the kindness of Dr. L. A. Sweet, Parke, Davis & Co.

‖ Obtained from The American Type Culture Collection. *M. lepræ* is of course of questionable identity.

¶ Obtained through the courtesy of Dr. Guy P. Youmans, Northwestern University Medical School.

** Obtained through the courtesy of Dr. William H. Feldman, Mayo Foundation.

antagonizes sulfonamide activity[5] and whole blood and serum up to 25%.

The antimycobacterial activity of 5-amino-2-butoxypyridine and its derivative is primarily bacteriostatic rather than bactericidal. (We hold the term bactericidal in its strictest sense, *i.e.*, complete sterilization of a sizable inoculum in a culture medium which, when free of inhibitory compounds, supports vigorous growth). Concentrations of 5-amino-2-butoxypyridine 1;000 times the minimum tuberculostatic concentration do not consistently render the system free of viable organisms.

The fact that these compounds are bacteriostatic rather than bactericidal and strikingly specific for the acid-fast group of organism suggest a mechanism of action involving the interference with some essential metabolic process common to species of Mycobacterium but which is lacking, has readily available alternatives or is nonessential in other genera of organisms.

[5] Lockwood, J. S., and Lynch, H. M., *J. A. M. A.*, 1940, **114**, 935.

15531

Bilateral Nephrectomy in Rats: Blood Chemistry, Longevity and the Effect of Aluminum Hydroxide.

J. A. ROSENKRANTZ.* (Introduced by M. Bruger.)

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In the hypocalcemic tetany of the uremic patient aluminum hydroxide reduces the tendency towards convulsions by elevating the serum calcium[1] presumably by preventing the absorption of the phosphate ion.[2] Subsequently, these studies were initiated in order to observe the effect of aluminum hydroxide gel on the survival time and on the blood levels of sulphates, phosphates, calcium, urea and cholesterol in rats following bilateral nephrectomy.

Method and Material. A total of 108 rats were used in this study. Fifty-one rats were employed in the longevity study of which 26 (16 males and 10 females) served as controls and 25 (15 males and 10 females) as experimental animals. The males weighed 400 to 500 g and the female rats 250 to 300 g. Both kidneys were removed by a transperitoneal approach through a midline ventral incision under ether anaesthesia. Sterile technic was utilized throughout. Each of the animals in the experimental group was given 5 ml of 4% aluminum hydroxide gel by stomach tube. An analgesic dose of ether was administered prior to the passage of the stomach tube in order to permit intubation without the use of a gag. The aluminum hydroxide was given once daily for 2 days prior to nephrectomy, then 4 to 6 hours after surgery, and thereafter once every 24 hours until the animals expired. The survival time of these rats was compared with the control group.

For the study of variations in blood chemistry, 28 non-nephrectomized male rats were anaesthetized with ether after which the abdominal aorta was exposed through a midline ventral incision. Blood was then aspirated from the aorta by means of a 26-gauge needle and a 2-ml hypodermic syringe. Seventeen male rats were nephrectomized and 48 to 50 hours postoperatively blood was withdrawn from the abdominal aorta as described above. In addition, 11 male rats were nephrectomized but were given aluminum hydroxide by stomach tube. These animals were also sac-

* Research Assistant in Medicine, New York Post-Graduate Medical School, Columbia University, New York City.

[1] Personal observation, unpublished data.

[2] Evans, G. T., and Flink, E. B., *Modern Med.*, 1945, **13**, 63; Fauley, G. B., Freeman, S., Ivy, A. C., Atkinson, A. J., and Wigodsky, H. S., *Arch. Int. Med.*, 1941, **67**, 563.

rificed 48 to 50 hours postoperatively in order to obtain blood from the abdominal aorta. The bloods were analyzed for serum inorganic sulphates, serum inorganic phosphates, serum calcium, whole blood urea and whole blood cholesterol.

Serum calcium was determined by the Clark-Collip[3] modification of the Kramer-Tisdall method and serum inorganic phosphates by the Lowenberg and Mattice[4] modification of the Benedict-Theis[5] method. Blood serum was analyzed for inorganic sulphates according to the method of Power and Wakefield.[6] The procedure of Myers[7] was used for the estimation of whole blood urea nitrogen. The whole blood cholesterol was determined by the method of Sackett.[8] Since the amount of blood withdrawn from the abdominal aorta of any one rat was frequently insufficient for complete studies, several samples were mixed thoroughly by gentle shaking and an aliquot portion of this pooled blood was used.

Results. Of 26 control rats, 14 or 54% expired on the 3rd day following bilateral nephrectomy while 12 (46%) died on the 4th day. The sex of the animal was not an influencing factor. In 25 nephrectomized rats receiving aluminum hydroxide, 12 (48%) expired on the 3rd day, 7 or 28% on the 4th day, while 20% lived beyond the control survival time (4 for 5 days and 1 for 6 days).

The average whole blood urea nitrogen of 28 normal rats was found to be 17.3 mg % (range 14-20.5), average serum calcium 9.5 mg % (range 8.4-10.4), average serum inorganic phosphates 6.9 mg % (range 4.5-9.2), average serum inorganic sulphates 2.97 mg % (range 2.70-3.49) and whole blood cholesterol 55.5 mg % (range 42-70). The 17 nephrectomized rats which did not receive aluminum hydroxide had an average urea nitrogen of 194 mg % (range 130-250), calcium 10.4 mg % (range 9.4-12.0), phosphates 8.1 mg % (range 6.5-10.8), sulphates 23.2 mg % (range 15-28.8) and cholesterol 70.8 mg % (range 60-96) 48 to 50 hours postoperatively. Bilateral nephrectomy, therefore, caused a marked retention of urea nitrogen and sulphate and a somewhat smaller rise in calcium, phosphate and cholesterol. The 11 nephrectomized rats receiving aluminum hydroxide showed an average urea nitrogen of 182 mg % (range 150-203), calcium 9.5 mg % (range 8.2-9.8), phosphates 7.9 mg % (range 6.1-9.4), sulphates 21.6 mg % (range 14.1-26.3) and cholesterol 65.5 mg % (range 60-69) 48 to 50 hours postoperatively. Apparently, the administration of aluminum hydroxide to bilateral nephrectomized rats was accompanied by slight decreases in each of the chemical constituents studied.

Discussion. The survival time of bilateral nephrectomized rats in this series is consistent with that of other investigations.[9] The increase of 20% in the longevity rate by the administration of aluminum hydroxide is difficult to explain, although it is conceivable that aluminum hydroxide may adsorb some of the toxic metabolites excreted into the intestinal tract (Adams).[10] It is universally appreciated that a moderate or severe acidosis develops in retention uremia;[11] the retention of the acid sulphate ion is probably the most important factor in the production of the acidosis. Aluminum hydroxide does not influence the acidosis directly since it is insoluble and nonabsorbable.[12] One may postulate that this aluminum compound interrupts, temporarily, the vicious cycle which might exist in nephritic acidosis, namely, the continued resecretion of high concentrations of

3 Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

4 Lowenberg, C., and Mattice, M. R., *J. Lab. and Clin. Med.*, 1930, **15**, 598.

5 Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, **61**, 63.

6 Power, M. H., and Wakefield, E. G., *J. Biol. Chem.*, 1938, **123**, 665.

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8 Sackett, G. E., *J. Biol. Chem.*, 1925, **64**, 203.

9 Page, E. W., and Ogden, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 511; Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, 1942, **136**, 577.

10 Adams, W. L., *Arch. Int. Med.*, 1939, **63**, 1030.

11 Marriott, W. M., and Howland, J., *Arch. Int. Med.*, 1916, **18**, 708; Holt, L. E., and McIntosh, R., *Holt's Diseases of Infancy and Childhood*, 10th ed., D. Appleton-Century, 1934.

12 Report Council on Pharmacy and Chemistry, *J. A. M. A.*, 1941, **117**, 1356.

acid into the stomach, by adsorbing total and free acid.[2,11,13]

The increase in inorganic acids of the blood is perhaps more important than the retention of nitrogenous products particularly insofar as acid-base balance is concerned. Denis[14] has demonstrated an increase of the blood sulphate level in patients with chronic nephritis and Macy[15] has shown an impaired clearance of sulphates in this disease. The present study shows that sulphate retention is appreciable in the nephrectomized rat.

No significant changes either in phosphates or serum calcium levels were noted in the nephrectomized rat. In the presence of prolonged acidosis and proteinuria (calcium adsorbed to protein) such as is found in chronic nephritics one might expect changes in serum calcium; in this study the element of time was too short and the factor of proteinuria absent.

Conclusions. 1. The longevity of the bilateral nephrectomized rat was 3 to 4 days. 2. The oral administration of aluminum hydroxide increased the survival time of 20% of nephrectomized rats to 5 or 6 days. 3. Bilateral nephrectomy resulted in a marked elevation of urea nitrogen and inorganic sulphate in the blood whereas the retention of inorganic phosphate, calcium and cholesterol was insignificant. 4. The oral administration of aluminum hydroxide in the bilateral nephrectomized rat produced a slight decrease in the degree of retention of each of the various blood constituents studied.

[13] Crohn, B. B., *J. Lab. and Clin. Med.*, 1929, 14, 610; Collins, E. N., Pritchett, C. P., and Rossmiller, H. R., *J. A. M. A.*, 1941, **116**, 109; Einsel, I. H., Adams, W. L., and Myers, V. C., *Am. J. Digest. Dis. and Nutrition*, 1934, **1**, 513; Rossett, N. E., and Flexner, J., *Ann. Int. Med.*, 1944, 21, 119.

[14] Denis, W., and Hobson, S., *J. Biol. Chem.*, 1923, 55, 183.

[15] Macy, J. W., *Arch. Int. Med.*, 1934, 54, 389.

15532 P

Reproduction in Chickens on Synthetic B-Complex Supplement.

THOMAS H. JUKES AND E. L. ROBERT STOKSTAD.
From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

Reproduction in rats on a diet containing the B-factors in synthetic form was previously reported.[1] However, it was not possible to rear chickens on a diet of this type until synthetic pteroylglutamic acid became available.[2] Day-old New Hampshire chicks were placed on the following diet: glucose (cerelose) 58.5 g, purified casein (Labco) 20 g, gelatin 8 g, calcium gluconate 5 g, cystine 0.4 g, choline chloride 0.2 g, inositol 0.1 g, bone ash 2 g, NaCl 0.6 g, KH_2PO_4 0.45 g, K_2HPO_4 0.6 g, $MgSO_4$ 0.25 g, $MnSO_4,4H_2O$ 0.05 g, ferric citrate 0.05 g, $CuSO_4,5H_2O$ 2 mg, $Al_2(SO_4)_3,18H_2O$ 1.6 mg, zinc acetate 1.4 mg, KI 0.6 mg, cobalt chloride 0.4 mg, nickel chloride 0.2 mg, calcium pantothenate 5 mg, niacinamide 5 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, thiamine HCl 1 mg, *p*-aminobenzoic acid 1 mg, pteroylglutamic acid 0.5 mg, *1-acetoxy-2-methyl-4-naphthyl* sodium phosphate 0.5 mg, (dl) biotin .04 mg, to which were added vitamin A 1500 U.S.P. units, vitamin D 200 A.O.A.C. units, mixed tocopherols 34 mg, dissolved in corn oil (Mazola) to a total of 3 g. The birds were kept in metal cages with wire floors. In this diet, the fat-soluble factors were fed in a crude form but the water-soluble vitamins, except inositol, were all synthetic and were fed at levels which appeared to be more than sufficient in each

[1] Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1940, 45, 625.

[2] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subba Row, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, 103, 667.

case, as judged by previous experiments and by results reported by other laboratories. Para-aminobenzoic acid is not known to be needed by chicks, but was fed as a precautionary measure. The calculated essential amino-acid content[3] of the diet satisfied the requirements of the chick for growth as described by Almquist and coworkers.[4] The chicks had an average weight of 1206 g at 10 weeks, and the group consisted of 2 males and 5 females. The chicks appeared normal in size, conformation and plumage. One of the males was observed to attempt copulation at 110 days of age. After the 155th day, 3 females and one male were kept in one group, and 2 females and one male in the other group. The males were rotated every 3rd day. Three of the females matured between the 151st and the 161st days of age, and the eggs were collected and incubated. On the 172nd day, an additional 1% of $CaCO_3$ was added to the diet to promote egg-shell formation. At 181 days of age the females averaged 2.26 kg in weight and the males 3.07 kg. The following record was obtained with the eggs:

Hen No.	1	2	3
No. of days	14	16	5
No. of eggs laid	8	9	4
No. of eggs incubated	6	4	3
Infertile	1	1	0
Dead embryo, estimated 1 to 7 days development	1	1	2
Dead embryo, estimated 17 to 21 days development	2	1	1
Live chicks hatched	2	1	0

The chicks appeared normal although they were small. They were placed on the same diet as their parents, without additional $CaCO_3$. They weighed an average of 28 g at hatching, and their average gain during the first month was 189 g.

Summary. Reproduction was obtained in chickens which were raised from hatching on a purified diet. The hatchability of the eggs was poor, but some apparently normal chicks were obtained.

3 Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods*, Springfield, Illinois, C. C. Thomas, 1945.

4 Almquist, H. J., *Fed. Proc.*, 1942, **1**, 269; Almquist, H. J., and Grau, C. R., *J. Nutrition*, 1944, **28**, 325; Grau, C. R., and Almquist, H. J., *Poultry Sci.*, 1944, **23**, 486.

15533

Antihistamine and Antianaphylactic Effect of Hetramine, a New Synthetic Pyrimidine Compound.

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From the Biological Research Laboratories of the Pyridium Corporation, Yonkers, N.Y.

There has been increasing evidence in recent years which indicates that some of the symptoms of anaphylactic shock and other hypersensitivities are due to the liberation of histamine during an antigen antibody reaction.[1-3] For this reason much investigation has been directed toward the discovery of a substance that can counteract the effects of histamine *in vivo*.[4-8] There has been additional evidence recently that substances having antihistamine properties can do much to

1 Dale, H. H., and Laidlow, P. P., *J. Physiol.*, 1910, **41**, 318.

2 Dragstedt, C. A., *Physiol. Rev.*, 1941, **21**, 563.

3 Code, C. F., *Ann. Allergy*, 1944, **2**, 457.

4 Bovet, D., and Staub, A. M., *C. R. Soc. de Biol.*, 1937, **124**, 547.

5 Staub, A. M., and Bovet, D., *ibid.*, 1937, **125**, 818.

6 Staub, A. M., *Ann. Inst. Pasteur*, 1939, **63**, 400 and 485.

7 Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharm. Exp. Therap.*, 1945, **83**, 120.

8 Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

TABLE I.
Acute Toxicity of Hetramine for Mice.

Dose mg/kg	Route of administration	No. of mice	No. dead	% dead
25.0	S.Q.	10	0	0
31.25	S.Q.	18	0	0
50.0	S.Q.	10	1	10.0
62.5	S.Q.	18	4	22.2
75.0	S.Q.	10	6	60.0
100.0	S.Q.	10	10	100.0
125.0	S.Q.	18	18	100.0
200	P.O.	15	0	0
250	P.O.	23	0	0
300	P.O.	20	4	20.0
400	P.O.	20	14	70.1
500	P.O.	13	10	77.0
600	P.O.	28	22	78.6

S.Q. = subcutaneously.
P.O. = orally.

Hetramine was studied for its antihistamine and antianaphylactic properties in experimental animals. It was found to have an unusually high degree of activity in preventing histamine-induced contractions of isolated intestinal strips. Similarly, it was found to be highly effective in blocking histamine-induced shock in the guinea pig and in preventing fatal anaphylactic shock in hypersensitive animals.

The acute toxicity of hetramine for mice was determined following subcutaneous and oral administration of the compound to respective groups of animals. The LD_{50} following subcutaneous administration is between 62.5 and 75 mg/kg of mouse weight. Following oral administration, the LD_{50} was determined to be approximately 300 to 400 mg/kg. The data showing these results may be seen in Table I.

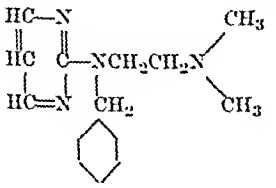
TABLE II.
Influence of Hetramine Upon Toxic Doses of Histamine.

No. guinea pigs	Hetramine pretreatment 30 min, mg/kg	Histamine mg/kg, intracardially	No. dead	% survival
6	1.0—I.P.	0.85	5	16.7
3	3.0—I.P.	0.85	3	0
6	4.0—I.P.	0.85	2	66.7
6	5.0—I.P.	0.85	1	83.3
7	6.0—I.P.	0.85	1	85.7
4	10.0—P.O.	0.85	3	25.0
5	20.0—P.O.	0.85	3	40.0
5	30.0—P.O.	0.85	3	40.0
5	40.0—P.O.	0.85	1	80.0
5	50.0—P.O.	0.85	0	100.0
8	Controls	0.85	8	0

I.P.—intraperitoneally
P.O.—per orally.

alleviate some of the symptoms associated with various types of allergies and other instances of hypersensitivity.

Hetramine which is N,N-dimethyl-N¹benzyl-N¹(a-pyrimidyl) ethylene-diamine,*



is a pyrimidine isostere of a pyridine compound which was found to have antihistamine activity.[8]

A preliminary experiment on the chronic toxicity of hetramine was carried out by testing the effect of continuous small daily doses in growing rats over a 35-day period. One group of 12 rat weanlings were put on a stock laboratory diet in which was incorporated a quantity of hetramine so that the rats consumed 50 mg/kg of rat weight per day. The concentration of hetramine in the food was adjusted daily to compensate for the growth of the rats and greater food consumption as the days passed, thus keeping the daily dosage to almost exactly 50 mg/kg per day. A second group of 12 weanlings were given a

* Syuthesized by H. L. Friedman and A. T. Tolstoouhov of the chemical laboratories of the Pyridium Corporation.

TABLE III.
Efficacy of Hetramine in Preventing Fatal Experimental Asthma in Guinea Pigs Exposed to Atomized Histamine.

Hetramine mg/kg, I.P.	No. guinea pigs	No. dead	% survived
0 controls	23	22	4.3
0.156	3	3	0
0.312	3	2	33.3
0.625	7	3	57.1
1.25	4	1	75.0
1.55	6	2	66.7
2.0	6	0	100.0
2.5	4	0	100.0

I.P.—intraperitoneally.

stock diet and the drug was administered subcutaneously, twice daily, 10 mg/kg at each dose. The doses were adjusted daily to the weight of each rat. Injections were given at 9:00 a.m. and 4:30 p.m. A third group of 12 weanling rats, serving as controls, were maintained on the stock diet and given no drug.

The animals were weighed every 2nd or 3rd day and hemoglobin determinations were carried out at intervals during the 35 days of the test. These doses of hetramine were tolerated without any effect upon the growth of the young rats and without any effect on the hemoglobin, the initial average weight being 25 g in all groups and the final weight averaged 127 g in the control and 125 and 123 g in the treated groups.

The antihistamine effect of hetramine on smooth muscle strips was demonstrated in an isolated muscle bath apparatus using strips of guinea pig ileum. Kymograph tracings of the muscular contraction were made on the addition of hetramine in amounts to bring its concentration in the bath to 1 γ per cc. Histamine solution was superimposed on the hetramine to bring its concentration to 1 γ per cc. The resulting contraction if any, was recorded on the kymograph and the muscle was then washed with fresh Ringer's solution and retested with 1 γ per cc of histamine. The absence of a contraction following the addition of histamine in the presence of hetramine indicated the antihistamine effect of the latter compound. The proof of histamine activity in the concentration used was shown by the immediate contraction of the muscle on the addition of histamine after having washed out the hetramine.

As little as 1/16 of 1 γ of hetramine counteracted the muscular contractions produced by 1 γ of histamine and partial inhibition was obtained in a ratio of one part of hetramine to 32 parts of histamine.

The *in vivo* antihistamine effect of hetramine was examined by pretreating guinea pigs with various doses of the compound to be followed in 30 minutes with toxic intracardial doses of histamine. 0.85 mg/kg of histamine intracardially was found to be uniformly fatal to guinea pigs. In Table II it is shown that pretreatment with 4.0, 5.0 or 6.0 mg/kg of hetramine is effective in preventing acute toxic death of the majority of animals tested. It is also demonstrated in Table II that oral dosage is similarly effective in this respect,

TABLE IV.
Antianaphylactic Effect of Hetramine in Guinea Pigs Sensitized to Horse Serum.

No. guinea pigs shocked with 1.0 cc horse serum I.C.	Pretreatment (20 min) hetramine mg/kg I.P.	Results Intensity of reaction following shocking dose No. pigs				
		++++	+++	++	+	0
11	0 controls	11	0	0	0	0
6	3.5	6	0	0	0	0
8	5.0	4	1	1	1	1
8	6.0	1	1	3	1	2

I.C.—intracardially.
I.P.—intraperitoneally.
++++—fatal shock.
+++—severe shock with recovery.
++—moderate reaction with recovery
+—slight reaction with recovery.
0—no symptoms

but must be increased about 10-fold.

Exposure to atomized histamine induces a severe and frequently fatal bronchoconstriction in the guinea pig. It was found that hetramine can prevent the fatal bronchoconstriction so produced by protecting the animals with varying doses prior to exposure to a histamine atmosphere. The chamber into which atomized histamine is introduced was constructed in accordance with the methods described by Loew, *et al.*[7] It was found that vapor from 5 cc of a 0.125% solution of histamine was almost uniformly fatal to unprotected animals. This mortality could be significantly and increasingly reduced with doses of 0.3 mg/kg to 2.5 mg/kg of hetramine administered intraperitoneally. The data on this experiment are shown in Table III.

The antianaphylactic effect of hetramine was determined by sensitizing guinea pigs through intraperitoneal injection of 0.25 cc normal horse serum 14 days prior to shocking doses of 1.0 cc horse serum administered intracardially. Control animals uniformly succumbed to the shocking dose with typical symptoms of fatal anaphylactic shock. Pretreatment with intraperitoneal doses of hetramine up to 3.5 mg/kg were ineffective under our conditions. When doses of 5 mg/kg were administered 20 minutes before the shocking dose of horse serum, 50% of the guinea pigs survived and 6.0 mg/kg reduced the severity of the reaction still further, 7 out of 8 animals surviving the shocking dose. The data are shown in Table IV.

The antihistamine effect of hetramine as demonstrated by the above laboratory experiments would indicate that this drug may have a beneficial effect on some of the varied symptoms associated with hypersensitivity and physical allergies. Clinical trials are in progress to determine the effect of this compound in a variety of conditions such as, vasomotor rhinitis, hay fever, urticaria, bronchial asthma, cold allergy and other physical allergies, serum reactions and contact dermatitis.

15534

On Breeding "Wild" House Mice in the Laboratory.

H. A. SCHNEIDER.*

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The white laboratory mouse may well be regarded as a classical example of the domestication of an animal at the hand of man. The consequences of that domestication have provided laboratory experimenters with a small docile animal the usefulness of which has been demonstrated so often as to be an established tradition. However, another consequence of that domestication has been the emergence of many genotypes distinguishable one from another by a variety of characters. This multiplicity of genotypes is a forceful reminder of the variation inherent in the species, and when, for example, a given diet produces a certain result in a certain laboratory strain of mice, we next may be expected to inquire whether (a) other genotypes will react similarly, or (b) all genotypes will do so, or (c) whether the wild species will do so. In many ways an investigation of the wild type would seem to have the greatest interest and meaning. This necessitates a laboratory supply of the wild genotype obtained either by trapping the numbers required, or by breeding them in the laboratory. For many purposes the latter method is the more preferable.

Recent investigations in the nutritional aspects of experimental epidemiology[1] have raised this question of host genotypes among

* With the technical assistance of Mr. S. A. Greenhalgh.

1 Schneider, H. A., and Webster, L. T., *J. Exp. Med.*, 1945, **81**, 359.

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2.0	6	0	100.0
2.5	4	0	100.0

I.P.—intraperitoneally.

stock diet and the drug was administered subcutaneously, twice daily, 10 mg/kg at each dose. The doses were adjusted daily to the weight of each rat. Injections were given at 9:00 a.m. and 4:30 p.m. A third group of 12 weanling rats, serving as controls, were maintained on the stock diet and given no drug.

The animals were weighed every 2nd or 3rd day and hemoglobin determinations were carried out at intervals during the 35 days of the test. These doses of hetramine were tolerated without any effect upon the growth of the young rats and without any effect on the hemoglobin, the initial average weight being 25 g in all groups and the final weight averaged 127 g in the control and 125 and 123 g in the treated groups.

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As little as 1/16 of 1 γ of hetramine counteracted the muscular contractions produced by 1 γ of histamine and partial inhibition was obtained in a ratio of one part of hetramine to 32 parts of histamine.

The *in vivo* antihistamine effect of hetramine was examined by pretreating guinea pigs with various doses of the compound to be followed in 30 minutes with toxic intracardial doses of histamine. 0.85 mg/kg of histamine intracardially was found to be uniformly fatal to guinea pigs. In Table II it is shown that pretreatment with 4.0, 5.0 or 6.0 mg/kg of hetramine is effective in preventing acute toxic death of the majority of animals tested. It is also demonstrated in Table II that oral dosage is similarly effective in this respect,

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6	3.5	6	0	0	0	0
8	5.0	4	1	1	1	1
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++++—fatal shock.
+++—severe shock with recovery.
++—moderate reaction with recovery
+—slight reaction with recovery.
0—no symptoms

7 of them for a period of 30 days. No evidence of oestrus was observed during this period. A variety of fresh green foods and a mixture of grass seeds fed as dietary supplements failed to bring the mice into oestrus.

Breeding Achieved. In May, 1944, we received a gift of 4 male and 4 female wild house mice from Dr. L. V. Heilbrunn of the University of Pennsylvania, at Philadelphia. (These mice were subsequently incorporated into our foundation stock). Dr. Heilbrunn informed us that these mice were the progeny of some wild house mice bred in his laboratory as pets. Comparison of the conditions under which these mice bred and our own failed indicated that, apart from dietary and bedding differences, the Philadelphia mice, in addition, had access to an exercise wheel. Breeding success in our own laboratory for all of our captured females was suddenly and finally achieved in July, 1944, following the introduction of exercise wheels. From that time forward we have been able to breed wild house mice at will and have prepared about 1,000 mice for experimental purposes. In this connection it is interesting to note that in their studies on the factors affecting the breeding of the field mouse *(Microtus agrestis)*, Baker and Ranson[4] recorded that their cages included a "revolving wire wheel" and that "the mice made frequent use of the wire wheels." With such cages these investigators have shown clearly that the breeding of *Microtus* proceeds best in an environment which supplies a daily period of incandescent illumination of 15 hours and that shortening the light day to 9 hours almost prevents reproduction. It is the female that is chiefly affected. At the moment our own experiments, conducted with a light day of 16 hours, do not permit a decision whether for *Mus. musculus* the effect of light is direct, or indirect through its effect on activity. However, it is a fact that in our experiments a 16-hour-light day, by itself, did not result in successful breeding. Experiments are now in progress to test the effect of activity with and without light.

The Complete Breeding Technic. The technic now followed in our laboratory for the breeding of wild house mice is as follows:

Environment. A single room (18′ x 15′) is devoted to the colony. A temperature of 75°F is maintained by thermostatic control, except for summer temperatures in excess of this. Temperature rises up to 85°F are tolerated without any change in the breeding performance, but temperatures in excess of 90°F result in breeding failure. Some preliminary experiments indicated that this failure was attributable to the males, probably due to the recognized effect of excessive heat on sperm viability. The animal room is lighted from 5 a.m. to 9 p.m. (16 hours) by 2-200 watt incandescent lamps controlled by a time-clock. All daylight is excluded.

Caging. The mice are bred, 1 male to 5 females, in galvanized iron boxes 9″ x 12″ x 9″ high with galvanized mesh covers through which the water bottle tubes are inserted. Centered and 3½″ from the top edge of the box the exercise wheel-drums are suspended on steel axles extending through the sides of the box. These exercise drums are lightly constructed of copper mesh for ease of turning and are 5″ in diameter and width. Four spokes permit the mice to enter the drum easily even while it is in motion, which is often. Revolution counters attached to these exercise drums indicated that in a 24-hour period a single pair of mice, male and female, will complete 40 to 60 thousand revolutions. At this rate, by free choice the mice have run approximately 10 to 15 miles per day. When a female is observed to be pregnant she is transferred to an individual galvanized box, 7″ x 10″ x 5″, to await her litter. The young are weaned at 4 weeks of age.

Bedding. Autoclaved pine shavings are used for bedding. The shavings must be of a size large enough so that a loose mass is formed on the floor of the cage into which the mice can penetrate and hide. This is important from the standpoint of mating behavior, for if smaller sized shavings were used which readily pack down and form a firm mass so that the mice were unable to hide in it easily, then, upon mating 5 females with 1 male it was observed that the females not in heat at a given time would attack the male and often kill him upon his attempting to mate with a female which was ready to accept him. No litters were ever obtained under such conditions even if the male sur-

laboratory mice feeding on different diets. In order to extend the experiments to include the wild genotype of *Mus musculus domesticus* we attempted to establish a laboratory colony of wild house mice. The difficulties encountered, the fact that others[2] have commented on their inability to breed wild house mice in the laboratory, and the absence of published accounts on the breeding of wild house mice have prompted us to report here the measures found necessary to establish a breeding colony with mice trapped "in the wild."

Foundation Stock. The wild house mice† which formed the base of our laboratory stock were trapped in spring-door traps in the fall of 1943. Every effort was made to insure that the mice trapped were not hybridized with domestic stocks which may have escaped from laboratories or dealers. An attempt was made, too, to secure representatives from different localities in and near New York City. Thus mice were trapped in a rural community (East Northport, Long Island), in New York City apartments, in suburban Woodside, Valley Stream, and in Long Island City, Long Island. All of these mice, and all subsequent additions to a total of 35, (17 males, 18 females) were subjected to a period of quarantine of 2 weeks before being admitted to the colony. During the quarantine period each mouse was subjected to 4 successive stool cultures testing for the presence of *Salmonella enteritidis* and *Salmonella typhimurium*. All of the 35 mice examined proved to be free of these pathogenic species and in the 2½ years elapsed since the inception of the wild mouse colony no mouse typhoid disease has made its appearance. Indeed, the mice have remained healthy throughout with no signs of overt disease among them. Of the 35 mice of the original foundation stock 6 (3 males and 3 females) are still alive more than 2 years later. As far as can be judged deaths have occurred mainly through fighting, and, less often, due to what can only be described as senility. No grossly visible tumors have been observed.

Breeding trials. The initial attempts to breed wild house mice were conducted with 8 captured females and 14 captured males. With the exception of 1 female, described below, all attempts met with failure for a period of 9 months extending from October 1943 to July 1944. There would be little gain now to list the details of these failures except to state that a variety of conditions were tried involving various sizes of caging and varying periods of illumination ranging from complete darkness to a light-day of 16 hours such as Baker and Ranson[4] found necessary for the breeding of the wild field mouse *(Microtus agrestis)*. In addition various beddings of wood shavings and peat moss were tried, as well as several stock diets all of which were satisfactory in supporting the prolific breeding of laboratory mouse stocks in adjacent cages in the same room at the same time.

During this period of breeding failure a single female, pregnant on admission to the laboratory on 10/11/43, was observed to deliver a litter of 3 mice on the following day. These she consumed. Subsequently this single female, during the time of breeding failure of 7 other captured females, gave birth to 3 litters following mating with captured males. The females among these litters, however, failed to breed with captured males even when 8 weeks of age and with perforate vagina.

Anoestrous of Wild Females. The failure of captured house mice to breed was traced to the failure of the females to achieve oestrus. Captured males were potent at all times and crossing with laboratory mouse females was easily done. The reciprocal cross failed, however, although tried on numerous occasions. Further evidence of the oestrous failure of the captured wild females was obtained by daily vaginal smear examination of

[2] Andervout, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

† The house mice of the northern United States are not truly "wild" types, but commensals (*Mus musculus domesticus*). Under favorable conditions these will adopt feral habits. The true wild type, from which *M. m. domesticus* was derived, is *M. m. wagneri*, the wild mouse of Russian Turkestan (Schwarz and Schwarz[3]).

[3] Schwarz, E., and Schwarz, H. K., *J. Mammalogy*, 1943, **24**, 59.

[4] Baker, J. R., and Ranson, R. M., *Proc. Roy. Soc. London*, B, 1932, **110**, 313; *ibid.*, B, 1932, **112**, 39; *ibid.*, B, 1933, **113**, 486.

56.8% to 70.2%. The average number of mice weaned per female rose in the same period from 3.69 to 4.08.

Handling of Wild Mice. All of the mice seen thus far in our colony have been as active and agile as the captured foundation stock. Special measures are necessary for handling them, for it is impossible to open a cage without having many escape. The simplest practical expedient is to perform all manipulations of the mice in a large box 18 to 20" deep with smooth sides. The escaping mice are thus confined to the larger box and may be recaught. With a little practice all of the ordinary manipulations of a mouse colony may be carried out quite easily.

Summary. The establishment of a breeding colony of wild house mice has been described. The inclusion of exercise drums in the breeding cages was found to be necessary.

15535 P

Effect of Oxygen on *P. lophurae* Infected Ducks.*

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It has been suggested that anoxia is an important factor in the mechanism of death in acute *P. falciparum* infection in man,[1] *P. knowlsi* infection in monkeys[2] and *P. lophurae* infection in ducks.[3] Furthermore it has been shown that ducks with malaria die sooner when placed in a decompression chamber than control birds kept at normal atmospheric pressure.[4] With the rapid destruction of red cells by the parasites the time may come during this disease when there is an insufficient number of red cells to carry a sufficient quantity of oxygen to supply the tissues of the host. The frequent intravenous injection of large quantities of duck red blood cells into birds that are moribund from malaria definitely prolongs their life.[5] In this study young ducks infected with *P. lophurae* have been placed in a chamber and given oxygen to determine its effect on the course of the infection.

In one experiment 10 ducks 15 days of age were inoculated intravenously with *P. lophurae* and put into the oxygen chamber. The oxygen within the tank was kept at approximately 50% concentration for 6 days at which time the amount of oxygen was increased to 75-85% for one day. Twenty similarly infected ducks were kept in batteries in the animal room. The time that death occurred in these 2 groups of ducks is shown in Experiment 1, Table I. A second group of 25 ducks 18 days of age were inoculated intravenously with *P. lophurae* infected blood. On the 4th day of the infection 10 of these were put into the oxygen chamber. The concentration of oxygen was kept at approximately 50% for 18 hours and then increased to 75% and ultimately to 85% during the following 24 hours. The 15 ducks in the control group were kept in the battery in the animal room. The time at which these birds died is shown in Experiment 2, Table I. In the third experiment 20 ducks 18 days of age were put into the oxygen chamber on the 4th day of

* This work was supported by a grant from the John and Mary R. Markle Foundation to the Department of Pathology. The oxygen was supplied by The Linde Air Products Company.

Research Paper No. 599, Journal Series, University of Arkansas.

[1] Rigdon, R. H., *Am. J. Hyg.*, 1942, **36**, 269.

[2] Rigdon, R. H., and Stratman, Thomas W. K., *Am. J. Trop. Med.*, 1942, **22**, 329.

[3] Rigdon, R. H., *Am. J. Trop. Med.*, 1944, **24**, 371.

[4] Rostorfer, H. H., and Rigdon, R. H., *J. Lab. and Clin. Med.*, 1945, **30**, 860.

[5] Rigdon, R. H., and Varnadoe, Nona B., *Am. J. Trop. Med.*, 1945, **25**, 409.

TABLE I.
Effects of Peat Moss and Exercise on the Breeding of Wild House Mice on a Diet of Whole Wheat Flour, Dried Whole Milk, and Salt.
(16-hr light-day.)

Diet No.	Diet description	Exercise drums	Within 60 days after mating: No. of females bred	No. of litters	Pregnancies
					%
100	Whole wheat, dried whole milk, NaCl	0	16	2	13
100	" " " " " "	+	13	11	85
259	Diet 100 plus 10% peat moss	0	15	6	40
259	" " " " " "	+	13	13	100

vived. This behavior problem could be solved either by mating the mice in single pairs, in which case the physical nature of the bedding was unimportant, or, preferably, by providing the shavings bedding in a state which allowed the mating pair to hide. Peat moss will serve as well as shavings.

Diet. At the time of the first breeding success in July 1944, following the introduction of exercise drums, all of the captured mice on hand were on a stock diet of ⅔ whole wheat, ⅓ dried whole milk and 1% NaCl (Diet 100). The bedding was peat moss. It was found that equally good results could be obtained by using pine shavings as bedding and adding 10% ground peat moss to the diet (Diet 259). This satisfactory, if unorthodox, diet served well in bringing up the numbers of the breeding stock at the same time that progeny were taken in large numbers for other experiments. At the present we are able to breed these wild mice very well on Diet 100 without the addition of peat moss. A recent experiment to test the effects of peat moss in the diet, as well as demonstrating the rôle of the exercise drums, is summarized in Table I. The females mated in this experiment were assembled into the experimental groups by dividing litter mates. There is thus no reason to attribute the obvious differences in breeding performance due to the presence or absence of exercise drums to genetic differences. The exercise effect is statistically significant; for Diet 100, $P<0.001$; for Diet 259, $P<0.01$. The effect of peat moss in improving the breeding performance, while suggestive, is not demonstrated with the same statistical adequacy ($P>0.1$).

It will be noted that on Diet 100, in the absence of an exercise drum, there were 2 pregnancies out of 16 mice bred. These 2 mice, in the absence of peat moss and exercise, probably came into oestrus by virtue of a genetic constitution which permitted this event in the absence of the environmental factors found necessary for the great number of wild house mice represented here. Such genetic variation would serve as the basis of selection of stocks independent of the environmental factors we have described as necessary, and by such means, to extend the argument, laboratory stocks may very well have been derived.

Breeding Plan. In order to maintain genetic heterogeneity the wild mouse colony has been bred in a manner so that closely related mice never form breeding pairs, but instead are crossed within the limits of the stock as much as possible. It is true, of course, that various kinds of automatic selection are going on which are slowly changing the statistical character of the wild genotype first assembled in the colony. Thus, those females which remain sterile under the conditions of the laboratory, in spite of the exercise drums, cannot contribute to the genetic composition of the succeeding generation. Such sterile genotypes will slowly be diminished in frequency. Using the per cent of pregnancies begun during the first 30 days following mating as an index of breeding performance, during the period of January 1945 to June 1946, such pregnancies rose from

blood for the different examinations. A few of the malarial infected birds were kept in the chamber and no smears were made. The fact that these ducks survived for a longer time when given oxygen would lend support to the opinion that anemic anoxia is a significant factor in the mechanism of death in the acute forms of malaria. The results of this study would indicate that the acute forms of this disease should be treated with a plasmodicial drug and also blood transfusions and oxygen should be given to combat the anoxemia.

Summary. It bas been sbown that ducks injected with *P. lophurae* survive for a longer time when placed in a chamber with 75 to 90% oxygen than the controls kept in batteries in the animal room. These observations support the opinion that anoxia is a significant factor in the mechanism of death in the acute forms of malaria.

15536

Inability of Penicillin to Neutralize Dick and Schick Toxins.

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The effect of penicillin upon the toxins of various bacteria is not as yet definitely established. Neter[1] showed that penicillin did not modify the action of tetanus toxin on mice. On the other hand, Boor and Miller[2,3] demonstrated that endotoxins obtained from meningococci and gonococci caused the death of fewer mice when the mice also received injections of penicillin. Meads and his associates,[4] as well as we,[5] have observed shortening of the course of scarlet fever in most of the patients who were treated with penicillin. The fever and toxicity diminished as soon as adequate doses of penicillin were given. As a result of these clinical observations we became interested in determining whether penicillin would neutralize the erythrogenic toxin of the hemolytic streptococcus. The experiments were later extended to include the toxin of the diphtheria bacillus. The results of these investigations are reported in the present paper.

Methods and Materials. One-tenth cubic centimeter of sodium penicillin in concentrations ranging from 10 to 1000 units per cc of isotonic salt solution were mixed with 0.9 cc of standard Dick or Schick toxins. In some instances the mixtures were injected immediately and in others they were first incubated for 4 or for 24 hours. The 2 controls used with each test were: (a) the same concentration of penicillin as that in the test dose, and (b) the standard toxins, diluted in salt solution to 90% of their usual strengths. Whenever the test mixtures were incubated prior to injection, the control materials were incubated for the same period of time. The skin reactions to Dick toxin were read 24 hours after intracutaneous injection and the Schick tests after 48 hours. The degree of erythema, edema and induration was used as criteria for judging the results.

Results. Dick Tests. Altogether 67 tests with mixtures of penicillin and Dick toxin were done on 63 subjects. The results are shown in Table I. The control tests containing penicillin in salt solution gave nega-

[1] Neter, Erwin, *J. Infect. Dis.*, 1945, **76**, 20.

[2] Boor, A. K., and Miller, C. P., *Science*, 1945, **102**, 427.

[3] Miller, C. P., and Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 18.

[4] Meads, M., Flipse, M. E., Barnes, M. W., and Finland, M., *J. A. M. A.*, 1945, **129**, 785.

[5] Hirsh, H. L., Dowling, H. F., and Sweet, L. K., *Ann. Int. Med.*, 1946, **25**, 78.

TABLE I.
Effect of Oxygen on *P. lophuræ* Infected Ducks.

	No. of ducks in each experiment No. of ducks dead					
	Exp. 1		Exp. 2		Exp. 3	
Time	10 ducks oxygen	20 ducks control	10 ducks oxygen	15 ducks control	20 ducks oxygen	21 ducks control
5th Day—						
8 a.m.					0	1
12			0	2	0	3
4 p.m.			0	5	0	7
8			2	10	4	12
12			5	11	5	13
6th Day—						
4 a.m.			7	11	5	13
8	1	7	8	12	7	16
12	2	11	8†	12†	10	18
4 p.m.	3	15			10	18
8	3	15			10	18
12	5	15			10	18
7th Day—						
8 a.m.	7	15			12	18
12	8*	15*			13‡	18‡

* Experiment discontinued, 2 ducks survived given oxygen, 5 of the controls survived.
† " " " 2 " " " " " 3 " " " "
‡ " " " 7 " " " " " 3 " " " "

infection and 21 were kept in the battery. The oxygen concentration was kept at approximately 50% for 24 hours after which it was increased to 85-90% for the following 2 days at which time the experiment was discontinued. The time that death occurred in these 2 groups of ducks is shown in Experiment 3, Table I. The data given in Table I indicate that ducks infected with *P. lophuræ* survive for a longer period when kept in an oxygen chamber containing 75 to 90% oxygen than similarly infected birds kept in batteries in the animal room.

The degree of parasitemia was followed in some of these infected ducks by counting the number of parasitized cells per 500 R.B.C. in the peripheral blood. Smears were stained with a combination of Wright's and Geimsa's stains. Our results indicate that the parasitemia is higher at the time of the peak of the infection in the birds kept in the presence of the high concentration of oxygen than it is in the control groups kept in the batteries. The total red cell counts were followed in a group of 6 young ducks kept in the oxygen chamber for 2 weeks with a concentration of approximately 50 to 75% of oxygen. These birds showed only a slight decrease in the total red cell count after being in the chamber for 5 days. The number of cells then gradually increased during a period of 48 hours and remained at a level slightly below normal for 6 days. After 2 weeks these birds were removed from the tanks and put into the regular batteries. There was no significant change in the red cell count during the following 6 days. Seven normal ducks 16 days of age were put into the chamber with an oxygen concentration of approximately 50%. After 2 days the blood from one of these birds was removed by cardiac puncture. The color index, volume index and relative cellular hemoglobin were determined. Blood was obtained from other ducks in the tank over a period of one week. Blood for the controls was obtained from similar aged birds kept in the battery. There occurred a slight increase in the color index and a slight decrease in the relative cellular hemoglobin of the ducks kept in the oxygen chambers over that of the controls.

The ducks were kept in the oxygen chamber continuously during these experiments except for the short intervals necessary to obtain

3. Penicillin injected into an area of rash of patients with scarlet fever did not cause blanching.

4. Dick positive patients receiving penicillin for an unrelated disease did not become Dick negative after a week of penicillin therapy.

5. It is concluded that penicillin has no neutralizing effect upon Dick or Schick toxin.

We wish to thank Dr. John H. Hanks for his interest and advice.

15537

Hypertonic Sodium Chloride Solution as Serum Diluent in Agglutination Tests with *Rickettsia burneti.**

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In the course of agglutination tests employing as antigen a killed suspension of the Balkan Grippe strain of *Rickettsia burneti*,[1] it was found that a prozone effect occurred with considerable frequency. In addition, the fine granular quality of the aggregates some-

TABLE I.
Effect of Varying Concentrations of Sodium Chloride, as a Diluent for Sera, on Agglutination of a Suspension of *Rickettsia burneti*.

Convalescent serum	Conc. of diluent % NaCl	Dilution of serum 1:4	1:8	1:16	1:32	1:64	1:128	1:256	Diluent control
1	0.85	4	4	4	2	1	0	0	0
	2.5	4	4	4	4	2	±	0	0
	5.0	4	4	4	4	2	1	0	0
	7.5	4	4	4	2	1	0	0	0
	10.0	4	4	3	2	0	0	0	0
	12.5	4	4	3	2	0	0	0	0
	15.0	4	4	3	1	0	0	0	0
2	0.85	4	4	4	2	2	±	0	0
	2.5	4	4	4	4	2	1	0	0
	5.0	4	4	4	4	2	1	0	0
	7.5	4	4	4	2	1	0	0	0
	10.0	4	4	3	2	0	0	0	0
	12.5	4	4	3	2	0	0	0	0
	15.0	4	4	2	1	0	0	0	0
3	0 85	4	4	4	4	3	±	0	0
	2.5	4	4	4	4	4	2	0	0
	5.0	4	4	4	4	3	2	0	0

0—No agglutination.
±, 1, 2, 3—Increasing degrees of agglutiuation.
4—Complete agglutination.

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1 Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1916, 44, 110-122.

TABLE I.

Effect of Penicillin upon Erythrogenic Toxin of *Streptococcus hemolyticus* as Determined by Dick Tests.

Conc. of penicillin in the test mixtures (units per cc)	Period of incubation (hr)	No. of tests	Results of control injection (0.9 cc Dick toxin and 0.1 cc salt solution)		Results of test injection (0.9 cc Dick toxin and 0.1 cc penicillin solution)	
			Negative	Positive	Negative	Positive
10	0	6	2	4	3	3
100	0	21	11	10	14	7
1000	0	18	3	15	3	15
100	4	3	2	1	2	1
1000	24	19	10	9	10	9
Total		67	28	39	32	35

TABLE II.

Effect of Penicillin upon Toxin of *C. diphtheriæ* as Determined by Schick Tests.

Conc. of penicillin in the test mixtures (units per cc)	Period of incubation (hr)	No. of tests	Results of control injection (0.9 cc Schick toxin and 0.1 cc salt solution)		Results of test injection (0.9 cc Schick toxin and 0.1 cc penicillin solution)	
			Negative	Positive	Negative	Positive
100	0	2	0	2	0	2
1000	0	2	0	2	0	2
10000	0	2	0	2	0	2
100	24	2	0	2	0	2
1000	24	2	0	2	0	2
10000	24	2	0	2	0	2
Total		12	0	12	0	12

tive results. Negative skin tests were observed with the Dick toxin control in 28 instances, while positive reactions were found in 39. When the combination of penicillin and Dick toxin was used, 32 were negative, and 35 were positive. The slight differences in the reactions observed are not considered significant. Incubation of the mixtures of penicillin and erythrogenic toxin did not modify the results.

Schick Tests. Twelve tests were performed on 6 subjects. The control tests with penicillin in salt solution were negative. As shown in Table II, all of the tests were positive regardless of whether the Schick toxin was mixed with isotonic salt solution or with penicillin, and regardless of whether the Schick toxin-penicillin mixtures were incubated or not.

Other Experiments with the Erythrogenic Toxin. Other methods were used to determine whether penicillin had any effect upon Dick toxin. In an experiment designed to ascertain whether penicillin would act similarly to antitoxin in the Schultz-Carlton reaction, 0.1 cc of a solution of penicillin containing 1000 units per cc was injected intracutaneously into an erythematous area of 3 patients with a florid scarlet fever rash. Blanching in this area did not occur before blanching of the general rash.

Ten patients receiving penicillin for an unrelated disease were found to be Dick positive. After they had received penicillin for one week and while penicillin was still being administered, a Dick test was again given. The test remained positive in each instance.

Summary and Conclusions. 1. When mixtures of penicillin and Dick toxin were injected intracutaneously into normal subjects along with parallel mixtures of Dick toxin and isotonic salt solution, no significant difference was observed in the number of positive Dick tests in the 2 series. Incubation of the mixtures, previous to testing, did not influence the outcome.

2. Similar results were obtained with Schick toxin.

as the one giving a final electrolyte concentration which was most nearly optimal. Additional experiments confirmed this finding. They indicate further that the prozone effect was reduced or eliminated when the 5.0% diluent was employed, and that a more dilute antigen could be used (Table II).

The application of these observations has made possible the use of a screen test consisting of one or 2 low-serum dilutions, for the rapid detection of rickettsial antibodies in large numbers of sera at a considerable saving of antigen.

Summary. The use of a 5.0% solution of sodium chloride as a serum diluent in agglutination tests with *Rickettsia burneti* reduced or eliminated the prozone effect and permitted the employment of a more dilute antigen.

15538

Induced *in vitro* Resistance of Staphylococci to Streptomycin and Penicillin.

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It has been recognized that certain strains of staphylococci acquire resistance to penicillin when grown in a medium containing this antibiotic.[1-6] Streptomycin[7] resistant strains can also be produced *in vitro* by exposure to this agent.[8-10]

The following study was undertaken to determine (1) the rate and degree to which various staphylococcal strains acquire resistance to streptomycin and to crystalline penicillin (G); (2) whether strains made resistant to penicillin simultaneously acquire resistance to streptomycin; (3) whether the resistant strains revert to their original sensitivity after repeated transfer in nontreated broth or when stored on dry ice for long periods of time; (4) if differences occur in the metabolic activity of the resistant and sensitive strains with respect to carbohydrate fermentation, and (5) to observe if changes in morphological characteristics occur after the organisms develop resistance to streptomycin.

Materials and Methods. The potency of the penicillin used was constant since crystalline penicillin (G) was used, however the streptomycin varied in potency from 250 to 600 units per mg. Bacto Brain Heart Infusion Broth was used as the liquid broth and standard F.D.A. agar served as the solid medium. Possible changes in the carbohydrate reactions of the cultures rendered resistant to streptomycin were investigated in Bacto Peptone Colloid Medium to which was added 0.002% phenol red and 1% of the carbohydrate. The media were inoculated from a 24-hour culture, incubated at 37°C and read after 24, 30, 48 and 120 hours.

Six strains of staphylococci obtained from different sources were used and designated as

[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

[2] Rommelkamp, C. H., and Maxtou, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

[3] McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

[4] Demerec, M., *Proc. Nat. Acad. Sci.*, 1945, **31**, 16.

[5] Todd, E. W., Turner, G. S., and Drew, L. G., *Brit. Med. J.*, No. 4386, 1945.

[6] Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

[7] Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

[8] Waksman, S. A., Reilly, H. G., and Schatz, A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 157.

[9] Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clinic*, 1946, **21**, 126.

[10] Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

TABLE II. Comparison of 0.85 and 5.0% NaCl Solutions, as Serum Diluents, on Agglutination of 2 Dilutions of Suspension of *Rickettsia burneti*.

Convalescent serum	Antigen dilution	0.85% NaCl — Serum dilutions 1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	5.0% NaCl — Serum dilutions 1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
1	1/2	2	3	4	4	4	2	±	0	0	4	4	4	4	3	2	1	0	0
	1/4	0	1	4	4	2	1	0	0	0	4	4	4	3	2	1	±	0	0
2	1/2	3	4	4	4	4	4	3	1	0	4	4	4	4	3	2	1	1	0
	1/4	2	4	4	4	4	4	2	1	0	4	4	4	4	3	2	1	±	0
3	1/2	2	3	4	4	4	3	1	0	0	4	4	4	4	4	2	1	0	0
	1/4	0	1	4	4	3	2	1	0	0	4	4	4	3	2	1	0	0	0
4	1/2	4	4	4	3	1	0	0	0	0	4	4	4	3	2	0	0	0	0
	1/4	1	2	3	3	±	0	0	0	0	4	3	2	1	1	0	0	0	0
5	1/2	3	4	4	4	3	2	1	0	0	4	4	4	3	2	1	0	0	0
	1/4	1	2	4	4	3	1	±	0	0	4	4	3	2	1	1	0	0	0
6	1/2	3	4	4	4	3	1	0	0	0	4	4	4	4	3	1	0	0	0
	1/4	2	4	4	4	4	1	0	0	0	4	4	3	2	1	1	0	0	0

0—No agglutination. ±, 1, 2, 3—Increasing degrees of agglutination. 4—Complete agglutination.

times caused uncertainty in determining end points. These difficulties were most often encountered with sera of high titer and with light antigen suspensions.

As is well known, the electrolyte concentration influences the rate of antigen-antibody reactions and the ratio in which antibody combines with antigen in flocculation and agglutination tests.[2] Accordingly, the effect of varying salt concentrations on the agglutination test with Balkan Grippe antigen was investigated.

The suspension of rickettsiae used as antigen was prepared in a density comparable to Barium Sulphate Standard No. 2.[3] The sera were obtained from patients in a laboratory outbreak of Q fever caused by the Balkan Grippe strain of *R. burneti*.[4] The effect of varying salt concentrations was determined by the use of a single antigen and 3 sera serially diluted in solutions of 0.85, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% sodium chloride, respectively.

The technic of the agglutination test[1] was as follows: sera were centrifugalized to remove particulate material and diluted serially in multiples of 2 in the respective saline solutions. To 0.2 ml of each serum dilution was added 0.2 ml of antigen dilution in 0.85% solution of sodium chloride. The tubes were incubated at 48°C for 10 minutes in a water bath, shaken for 3 minutes, returned to the 48°C water bath for 3 hours, and then placed at 4°C for 18 hours. The tubes were examined at the end of 1, 2, 3 and 21 hours. Readings were made with the aid of a concave mirror against a dark background.

The initial experiments indicated that agglutination occurred to a slightly higher titer when the sera were diluted with 2.5 and 5.0% solutions of sodium chloride (Table I). In addition, the rate of combination was somewhat more rapid with the 5.0% solution, than with the 2.5% solution. The 5.0% solution of sodium chloride was therefore selected

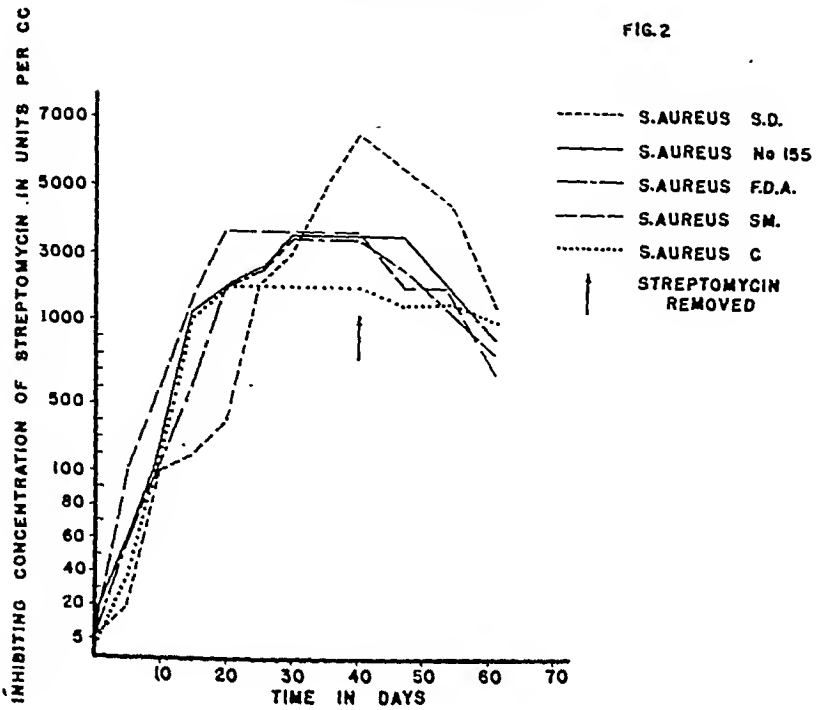
[2] Boyd, W. C., *Fundamentals of Immunology*, 1943, Interscience Publishers, Inc., New York.

[3] Wadsworth, A. B., *Standard Methods*, 1939, Williams and Wilkins Co., Baltimore.

[4] Commission on Acute Respiratory Diseases, *Am. J. Hyg.*, 1946, 44, 123-157.

ACQUIRED RESISTANCE OF VARIOUS STRAINS OF STAPHYLOCOCCUS AUREUS TO STREPTOMYCIN

FIG. 2



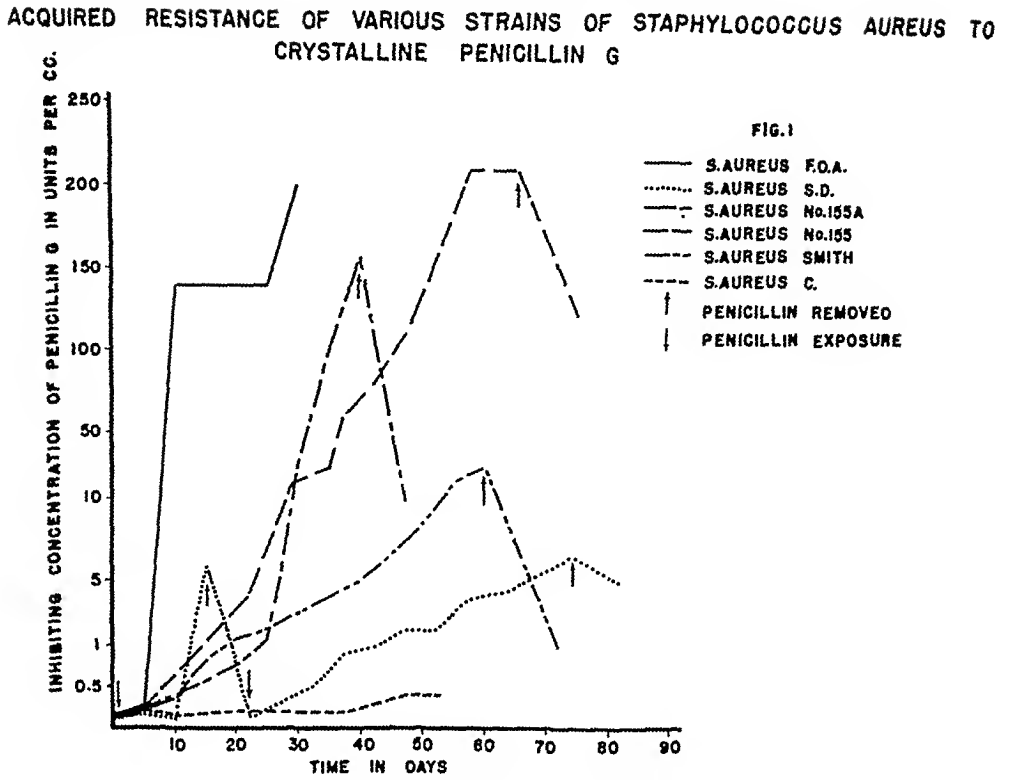
quired also varied as evidenced by the fact that one strain *(S. aureus F.D.A.)* developed a resistance of approximately 1000-fold in 10 days, whereas other strains required from 35 to 45 days to withstand the same increase in penicillin concentration.

A similar resistance developed when the original cultures were exposed to increasing concentrations of streptomycin (Fig. 2), but showed less variation than when exposed to penicillin. It seemed to occur more rapidly, but to a lesser degree than to penicillin. In all cases resistance increased rapidly until the organisms tolerated approximately 300 to 700 times as much streptomycin as originally. It was of interest to note that none of the resistant strains destroyed streptomycin indicating that increased resistance is not due to the formation of a streptomycin-destroying enzyme.

Regression of Induced Resistance. In order to determine whether a resistant strain would revert to its original sensitivity when cultured in broth free of penicillin, strain *S. aureus S.D.* which had acquired a resistance of 60-fold in 15 days was transferred daily in nutrient broth over a 7-day period. The results show that with this strain resistance was lost within 7 days (Fig. 1). Upon re-exposure to penicillin the culture became resistant to the same degree, requiring however, a longer period of time. Similar tests were performed with *S. aureus No. 155A, S. aureus Smith* and *S. aureus No. 155* after these cultures acquired an increased resistance of 1600-, 300- and 2100-fold respectively.

All of the penicillin-resistant strains tested showed some decrease in resistance upon transfer in nutrient broth.

When the streptomycin-resistant strains were transferred in a similar manner they also became more sensitive to the antibiotic. Over the 21-day period during which these strains were followed in broth they showed less variation in the rate at which resistance regressed than was observed with the penicil-



S. aureus Smith, S. aureus F.D.A., S. aureus No. 155, S. aureus No. 155A, S. aureus S.D., and *S. aureus C.*

Regression of induced resistance to the antibiotics was studied by freezing the fast strains and storing them on dry ice for from 9 to 12 months. Upon removal they were grown through several transfers before testing for resistance.

The sensitivity of each organism to the antibiotics was determined by the following method: A series of dilutions of the test agent in sterile distilled water was prepared in such a manner that they were 10 times more concentrated than the final dilution desired. To 1 cc of each dilution was added 9 cc of seeded culture medium containing a 10^{-5} dilution of a 24-hour culture of the test organism. The tubes were incubated at 37°C for 24 hours and examined for turbidity. The minimal concentration of the antibiotic in units per cc which resulted in complete inhibition of growth was taken as the inhibiting concentration.

Resistance was produced by transferring each culture into broth containing one-half of its inhibiting concentration of the antibiotic. After 5 such daily transfers, the bacteriostatic concentration for the culture was redetermined, and the drug concentration increased so as to equal one-half of the new inhibiting level. This procedure or a slight modification, was continued until no further significant change in resistance was evident. Control cultures were maintained by daily transfer in untreated broth.

Results. Induced Resistance. All of the cultures exposed to penicillin and streptomycin as described above decreased in sensitivity to the antibiotics. The degree to which resistance was acquired depended upon the strain and the duration of exposure. Thus, 3 strains of *Staphylococcus aureus* acquired a resistance to penicillin of at least 1500-fold within 10 to 52 days; whereas one culture became only 4 times more resistant when exposed in the same manner (Fig. 1). Furthermore, the rate at which resistance was ac-

TABLE I.
Carbohydrate Reactions of Streptomycin Resistant Staphylococcal Strains.

	S. aureus S. M.								*S. aureus F. D. A.*							
	Control Time in hr				Streptomycin Resistant Time in hr				Control Time in hr				Streptomycin Resistant Time in hr			
Carbohydrate	24	30	48	120	24	30	48	120	24	30	48	120	24	30	48	120
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
Lactose	—	—	—	—	—	—	—	—	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	—	—	—	+	+	+	+	+	±	+	+	+
Sucrose	+	+	+	+	—	—	±	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	—	±	+	+	+	+	+	+	+	+	+	+
	S. aureus Cameron								*S. aureus S. D.*							
Glucose	+	+	+	+	±	+	+	+	+	+	+	+	±	±	±	+
Lactose	+	+	+	+	±	+	+	+	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	±	±	+	+	+	+	+	+	±	±	±	+
Sucrose	+	+	+	+	±	+	+	+	+	+	+	+	±	+	+	+
Mannitol	—	—	—	—	—	—	—	—	+	+	+	+	±	+	+	+
	S. aureus No. 155															
Glucose	±	+	+	+	±	+	+	+								
Lactose	±	±	+	+	±	±	±	+								
Maltose	+	+	+	+	+	+	+	+								
Sucrose	+	+	+	+	+	+	+	+								
Mannitol	+	+	+	+	+	+	+	+								

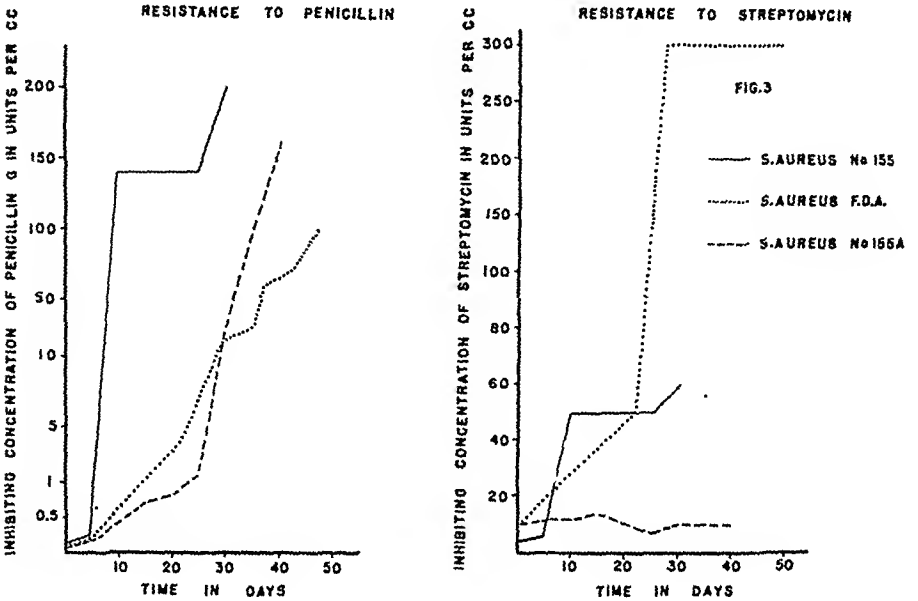
+ Acid. ± Some acid. — No acid.

marked differences in the appearance of the broth cultures or colony formation on agar, except for a loss of pigmentation by the resistant *S. aureus F.D.A.* and *S. aureus Smith.*

Carbohydrate Fermentation. At peak resistance the streptomycin-resistant staphylococcal cultures showed no change in the sugars fermented (Table I), but an appreciable increase in the time required for the fermentation was observed. Two weeks after the cultures had been returned to broth without streptomycin they were retested, and with the exception of *S. aureus No. 155* which continued slow in fermenting lactose, produced acid at the same rate as the control cultures.

Summary. 1. Under certain conditions strains of *S. aureus* develop resistance *in vitro* to streptomycin or to penicillin (G). 2. Greater variation occurs in the degree and rate at which resistance is acquired to penicillin than to streptomycin. 3. All of the streptomycin and penicillin-resistant strains studied showed some decrease in resistance upon transfer in broth free of the antibiotics. 4. Two of the penicillin-fast strains increased in resistance to streptomycin whereas the streptomycin-fast strains remained sensitive to penicillin. 5. Some streptomycin-fast staphylococcal strains show a change in pigment production and a reduction in the rate of carbohydrate fermentation.

ACQUIRED RESISTANCE TO PENICILLIN AND STREPTOMYCIN OF STAPHYLOCOCCAL STRAINS EXPOSED TO CRYSTALLINE PENICILLIN G



lin-resistant strains.

When stored on dry ice for from 9 to 12 months 3 of the 5 strains which had acquired resistance to penicillin were found to be more sensitive. The resistance of strains S.M., S.D. and No. 155A was reduced from 300-, 65- and 1600- to 40-, 3.5- and 70-fold respectively when stored for this period of time. On the other hand, strains F.D.A. and No. 155 remained practically unchanged. It is notable that the strains which retained their resistance were those which showed the greatest degree of increased resistance during exposure to penicillin. In contrast to the foregoing findings all streptomycin-fast strains retained their resistance to this antibiotic when stored at a low temperature for long periods of time.

Cross Resistance. During the period in which the staphylococci were being made resistant to penicillin they were also tested for sensitivity to streptomycin. A change in the inhibitory effect of penicillin on the streptomycin-fast strains was also investigated. Under these conditions it was observed that *S. aureus F.D.A.* and *S. aureus No. 155* while acquiring resistance to penicillin had simultaneously become 15 and 30 times more resistant to streptomycin. These strains had also shown the greatest increase in resistance to penicillin. Three additional strains did not show this increase in resistance. For simplicity the results observed with 3 representative strains are presented (Fig. 3). In contrast, all 5 streptomycin-resistant strains remained sensitive to penicillin.

Morphology. Cultures acquiring resistance to commercial penicillins have been reported to show morphological changes,[6,11,12] marked pleomorphism,[6,11] loss of pigmentation,[6,11] and slowing of metabolic reactions.[1,6,11,12] Similar morphological changes and pleomorphism as have been described by others were observed in the staphylococcal strains made resistant to crystalline penicillin (G).

Studies on the growth characteristics of the streptomycin-resistant strains showed no

[11] Gardner, A. D., *Nature*, 1940, **146**, 837.

[12] Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 210.

TABLE 1.

Case No.	Day of disease when treatment began	Avg incubation preserum isolation, days	Amt of serum administered between isolations	Interval between isolations	Avg incubation postserum isolation, days	Difference between pre- and post-serum incubation	Total amt serum administered (cc)
6	3	11½	39 iv—20 im	14 hr	14	Prolonged 2½ days	239
11	4	11½	58 iv	15 "	14*	" 2½ "	58
13	4	11½	51 iv	19 "	20*	" 8½ "	51
14	4	10	20 iv—37.5 im	16½ hr	12	" 2 "	218
15	4	13	126 iv	5 min	17*	" 4 "	512
16	4	10	85 iv	11 hr	12*	" 2 "	491
19	5	7	40 iv—40 im	Immediately after	11*	" 4 "	120
20	5	12	78 iv	1st isol, 21 hr	15	" 3 "	78
				2nd isol, 114 hr	10	Reduced 2 "	
22	5	10½	30 iv—60 im	7 hr	16*	Prolonged 5½ "	260
24	6	11½	59 iv	3 min	9½	Reduced 2 "	59
25	6	12	40 iv—38 im	7 hr	14½	Prolonged 2½ "	238

* One guinea pig of each of these groups did not react when observed 21 days.
The case numbers employed here correspond to those used by Yeomans, Snyder and Gilliam.[1]
Strain isolation for Case 6 and 13 is incorrectly recorded by Yeomans, Snyder and Gilliam.[1]

infectious material into guinea pigs. Finally, there may be a rickettsiostatic effect of the serum upon the circulating agent. The precise reason is not known for antibody studies were not made on these 5 specimens of guinea pig convalescent serum.

Seven of the cases (6, 14, 15, 16, 19, 22 and 25) received further doses of serum after the second isolation of the agent and hence, it is impossible to determine the possible effect of the early administration of serum upon the clinical course of the disease. However, Cases 11, 13, 20 and 24 were of particular interest since the total amount of serum administered occurred between the 2 strain isolations.

Cases 11 and 13 were recorded[1] as being of moderate severity, showing slight prostration, central nervous system involvement, cardiovascular changes or mild complications. The only effect observed after the administration of 58 cc and 51 cc of serum, respectively, was to prolong the incubation period in the guinea pigs when isolations were made 15 and 19 hours after serum treatment. Case 24, who received 59 cc of serum, was characterized as being a severe typhus with definite prostration, central nervous system involvement, cardiovascular changes or serious complications (nitrogen retention). When an isolation was attempted 3 minutes after the administration of serum, the period of incubation was reduced from what was found for the preserum isolation. In Case 20, which was characterized as being of such severe illness that a fatal outcome was expected at some point in the clinical course, the administration of 78 cc of serum had a slight effect upon the agent when isolation was made after 21 hours and none after 114 hours.

Since a favorable effect of the serum on the course of the illness was obvious from the clinical observations, the question arises as to what this effect may be attributed. It is evident that the serum did not have a rickettsiocidal effect upon the agent, for in 11 instances we were able to isolate the agent after the administration of serum. The serum may have had a rickettsiostatic effect. In favor of this hypothesis would be the prolonged incubation period in the guinea pigs in the postserum isolations. The final and

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Effect of Concentrated Hyperimmune Rabbit Serum on Circulating Agent in Louse Borne Typhus.

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Immune serum has been used by a number of workers in the treatment of rickettsial diseases. A recent report by Yeomans, Snyder and Gilliam reviewed the previous work and reported upon the use of a concentrated hyperimmune rabbit serum in the treatment of 25 cases of epidemic typhus fever studied in Cairo, Egypt.[1] These authors concluded that hyperimmune antityphus rabbit serum had a favorable therapeutic effect on the course of typhus if treatment was started in the first 3 days of the disease. Although serum treatment seemed to have reduced the mortality in the group of patients who received serum on the 4th, 5th, or 6th day of illness, the value of serum therapy for late cases could not be determined.

Of the 25 cases studied by Yeomans, Snyder and Gilliam, strains of epidemic typhus were isolated in guinea pigs by the authors in 20 instances. In 11 of these cases, attempt at strain isolation was made immediately before and again at variable times after the administration of the serum. Since the method employed for strain isolation was identical in each instance, it was possible to ascertain the effect of the serum upon the circulating agent. Furthermore, the nature of this effect could be determined, within certain limits, by comparing the period of incubation in the inoculated guinea pigs in the pre- and postserum isolations.

Method. Thirty cc of blood were withdrawn from the patient. The blood was kept in ice and usually was inoculated into guinea pigs within 2 hours after withdrawal. After coagulation, the serum was removed and the clot was ground up in a mortar with alundum. This material was then suspended in 10 cc of physiological saline. After the alundum sedimented, 5 cc of the supernatant fluid were inoculated intraperitoneally into each of 2 male guinea pigs. The temperatures of the guinea pigs were taken for several days prior to inoculation in order to obtain the normal level and for 21 days after. A temperature of 104°F or higher was considered as fever.

Table I records the pertinent data in this study. These data indicate that in 11 cases of epidemic typhus fever treated with hyperimmune rabbit serum, it was possible to isolate the agent in guinea pigs immediately before and again at variable periods after the administration of serum. This was true when the postserum isolations were made immediately after and up to 114 hours after the administration of serum. Even though circulating agent was still present after the administration of serum, some deleterious effect of the serum upon the agent was evident as judged by the prolongation of the period of incubation in the postserum isolations. There were 2 exceptions. In Case 24, blood was drawn for postserum isolation 3 minutes after the administration of serum. The incubation period in the inoculated guinea pigs was reduced 2 days. In Case 20, in an isolation made 21 hours after the administration of serum the period of incubation was prolonged by 3 days, while with the isolation made after 114 hours, this period was reduced 2 days. It should be noted that in 6 cases one guinea pig each in the postserum isolations failed to react when observed over a 21-day period. This failure to react may have been due to an inapparent infection which is characterized by no evident symptoms of disease but is followed by the appearance of specific antibodies, or by a missed infection characterized by no symptoms, or the appearance of specific antibodies.[2] These possibilities may occur normally after the inoculation of

* Member, United States of America Typhus Commission.

1 Yeomans, A., Snyder, J. C., and Gilliam, A. G., *J. A. M. A.*, 1945, **129**, 19.

2 Plotz, H., Wertman, K., and Bennett, B. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **41**, 76.

TABLE I.
Summary of Hog Cholera Virus Passages in Rabbit Spleen.

Rabbits				Swine	
Rabbit passage*	No. inoculated	No. with febrile reaction	Highest temp, °F	No. injected	Results
1	7	4	104.8		
3	2	2	104.7		
5	4	3	104.6		
6	3	3	105.2	2	1 sacrificed when sick on 6th day. 1 survived with development of immunity (see text).
8	4	3	105.1	2	2† died on 18th day with typical hog cholera lesions.
9	4	3	104.8	1	1† died on 18th day with typical hog cholera lesions.
11	4	2	105.6	2	2 survived. 1 showed delayed febrile reaction.

* Refers to number of passages counting from the 6th alternating pig-to-rabbit to pig-to-rabbit passage.

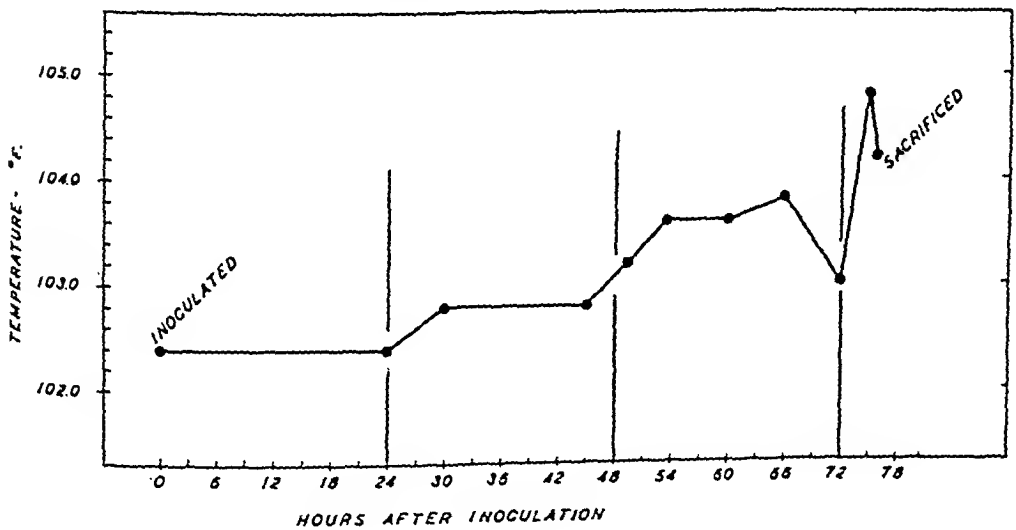
† Normal swine placed in the same pen with these animals contracted hog cholera and died from the disease.

The presentation of experimental data is the subject of this report.

Material and Methods. The hog cholera strain was the stock virus used by the Lederle Laboratories in St. Joseph, Missouri* for the production of hog cholera virus vaccine and immune serum. Pigs were bought from local farmers whose premises were known to be free from hog cholera and who employed no hog cholera vaccines. The pigs were obtained immediately after weaning and care was taken to ascertain that none of the animals were exposed to hog cholera or were sick prior to purchase. Upon arrival in this laboratory, the pigs were kept in strict quarantine for

* We are indebted to Mr. Frank Cooper, of the St. Joseph plant, for his cooperation in the initial stages of this work.

FIG. 1
TEMPERATURE CURVE OF RABBIT
INJECTED WITH THE EIGHTH RABBIT SPLEEN-PASSAGE VIRUS



most likely hypothesis is that the serum exerted beneficial clinical effect because of an antitoxic effect. It has been shown that a "toxic factor" is associated with the epidemic and murine strains of typhus rickettsiae cultivated in yolk sac cultures and that this "toxic factor" can be neutralized specifically by convalescent serum.[3-6] This may explain why such relatively small amounts of serum as were employed in Cases 11, 13, 20 and 24 exerted a beneficial clinical effect. The hyperimmune rabbit serum used in these cases was prepared with infected yolk sac cultures.

Conclusion. 1. In 11 cases of epidemic typhus treated with hyperimmune rabbit serum, strains were isolated immediately before and again at variable periods after the administration of serum. 2. The only effect observed was that in the postserum isolations the period of incubation in the inoculated guinea pigs was usually, but not always, prolonged. 3. While the effect of the serum upon the agent may be rickettsiostatic, it is believed that a more likely explanation of the favorable clinical effect was due to its antitoxic effect.

[3] Gildemeister, E., and Haagen, E., *Deut. Med. Wchschr.*, 1940, **66**, 878.

[4] Bengtson, I. A., Topping, N. H., and Henderson, R. G., Nat. Inst. of Health, Bull. No. 183, 1945, p. 25.

[5] Henderson, R. G., and Topping, N. H., Nat. Inst. of Health, Bull. No. 183, 1945, p. 41.

[6] Hamilton, H. L., *Am. J. Trop. Med.*, 1945, **25**, 391.

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Propagation of Hog Cholera Virus in Rabbits.

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The literature in regard to negative results in attempts to transmit hog cholera virus to hosts other than swine, is too voluminous to be cited. The only evidence of actual transmission of the virus in other hosts has been furnished by Zichis[1] who was able to propagate the virus for 10 passages in domestic sheep. Cultivation of the virus in hog tissue outside the body of the animal has been reported by Hecke[2] and TenBroeck.[3]

Even though all evidence indicated that hog cholera would be a difficult virus to adapt to other hosts, it seemed that in view of the knowledge gained with other viruses—such as: poliomyelitis,[4] dengue fever,[5] Colorado tick fever[6,7] and rinderpest,[8-11] the problem should be reinvestigated.

For some time attempts have been made in this laboratory to adapt hog cholera virus to some host other than swine. In accordance with the experience of most previous investigators most of our attempts ended in failure. Recently, however, by using an alternating passage method similar to that reported by Baker[10] in his work with rinderpest virus in rabbits, we have obtained results indicating that hog cholera virus may be successfully passed for a number of generations in rabbits.

[1] Zichis, J., *J. Am. Vet. Med. Assn.*, 1939, **95**, 272.

[2] Hecke, F., *Zentralbl. Bkt. Abt. I, Orig.*, 1932, **126**, 517.

[3] TenBroeck, C., *J. Exp. Med.*, 1941, **74**, 427.

[4] Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 2302.

[5] Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, **101**, 640.

[6] Florio, L., Stewart, M. D., and Mngrage, E. R. *J. Exp. Med.*, 1944, **80**, 165.

[7] Koprowski, H., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 320.

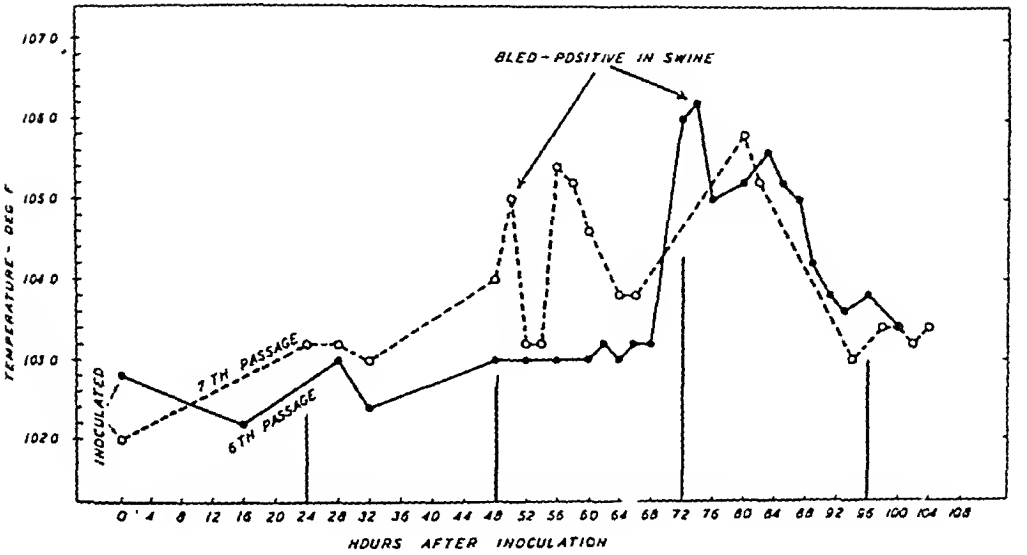
[8] Nakamura, J., Wagatuma, S., and Fukusho, K., *J. Jap. Soc. Vet. Sci.*, 1938, **17**, 185; English Summary, pp. 25-30.

[9] Kunert, H., *Dtsch. tierarztl. Wschr.*, 1938, **46**, 487.

[10] Baker, J. A., *Am. J. Vet. Res.*, 1946, **7**, 179.

[11] Shope, R. E., Griffiths, H. J., and Jenkins, D. L., *Am. J. Vet. Res.*, 1946, **7**, 135.

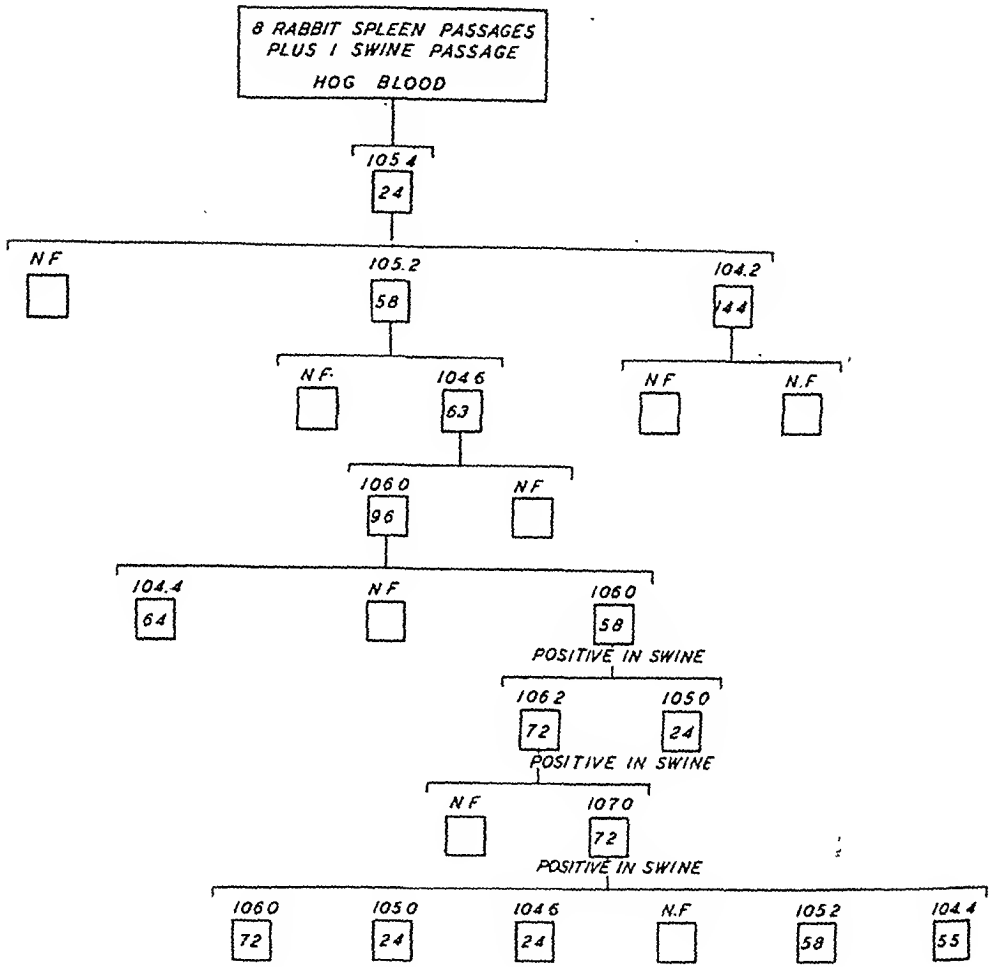
FIG 3
TEMPERATURE CURVES OF RABBITS
INJECTED WITH THE SIXTH AND SEVENTH RABBIT BLOOD-PASSAGE VIRUS



6th day after inoculation with hog cholera virus, was made into a 10% suspension in saline in a TenBroeck grinder. The suspension was centrifuged for 5 minutes at 1000 r.p.m. and 2 ml of the resulting supernatant were inoculated intravenously into a rabbit. The rabbit showed no elevation of body temperature but was sacrificed on the 3rd day after inoculation and a 10% suspension of its spleen inoculated into a pig. The latter showed fever up to 105-106°F on the 2nd, 3rd and 4th days after inoculation and was sacrificed on the 5th day. A suspension of its spleen, in turn, was injected into a rabbit. The virus was transferred by means of this technic of alternation between the hog and one or 2 rabbit passages until it had been carried through 6 intermittent rabbit passages. Out of 7 rabbits injected with the 6th passage virus, 3 showed slight elevation of temperature on the 2nd day after inoculation, and were sacrificed at that time. Their spleens were pooled and a suspension of them was injected intravenously into another group of rabbits. These animals also reacted with a slight fever and were sacrificed in turn, and the virus was thus transferred for 2 more rabbit passages. At this stage, none of the rabbits showed increased temperatures on routine checking and no virus was detected in their spleens by subinoculation of swine. It thus became obvious that the virus was either merely transferred from one rabbit to another without proliferation or that the technic of taking temperatures only twice during the daytime, following the technic used by Baker[5] in his work with rinderpest virus, was inadequate. In order to test the latter possibility, a rabbit spleen suspension, representing the 3rd rabbit passage, was injected into 3 rabbits, temperatures of which were taken every 2-3 hours throughout the 24-hour period. All 3 rabbits reacted with a febrile response on the 2nd night after inoculation. Curiously enough the elevated temperature lasted only 2-3 hours after which it started to drop. The animals were sacrificed during this febrile period and a pool of their spleens when injected into swine proved to contain hog cholera virus. By adopting the above technic of taking temperatures every 2 or 3 hours, day and night, the virus was carried for 12 continuous rabbit passages by spleen transfer. Febrile response was the only sign of infection in the rabbits. The data are summarized in Table I. A

FIG. 2

SUMMARY OF HOG CHOLERA VIRUS PASSAGES IN RABBIT BLOOD



- LEGEND -

54 - - FIGURES SHOW HOURS AFTER INOCULATION - BEFORE RABBITS WERE BLED

104.0 - - FIGURES SHOW TEMPERATURES AT THE TIME THE RABBITS WERE BLED

N.F. - - NO FEBRILE RESPONSE OBSERVED

2 weeks and released for experimental use only if signs of disease were absent in the entire lot. Inoculated pigs were housed in individual pens under strict isolation so that any possibility of cross-infection was ruled out. White rabbits of the "New Zealand" strain were used throughout the work. The animals were 5-8 lb. in weight and were kept individually in cages. The pigs were inoculated intramuscularly in the groin and the rabbits were inoculated in the marginal ear vein. Rectal temperatures were taken on the animals twice daily, at 8:30 a.m. and 4:30 p.m., unless otherwise stated.

Experimental. Infected hog spleen, derived from an animal which was sacrificed on the

sage transfers promptly at the time the febrile response occurred.

It may be of interest to mention here that other investigators[10,12-16] have used the concept of alternating passages to maintain, modify, reactivate or adapt viral or rickettsial agents to other hosts or tissues.

It is conceivable that it may not be essential to use the alternating passage method to pass hog cholera virus in rabbits since in work with rinderpest virus, Nakamura and colleagues[5] were able to adapt the virus to rabbits and maintain it for 166 consecutive serial passages without going back to the original host. Edwards[17] also reported success in maintaining rinderpest virus in rabbits for a period of at least 14 months by going directly from the calf to the rabbit and by making successive intravenous transfers in rabbits every 2-7 days. On the other hand Baker[1] found it necessary to use the alternating passage method to achieve the same object. The difference in results obtained by various investigators could be due to the fact that different breeds of test animals or different strains of virus may have been employed.

Summary. Hog cholera virus has been carried for 12 consecutive passages in rabbits by using infected rabbit spleen as transfer material. Starting from the 8th rabbit spleen passage, the virus was passed back to a pig for one passage and was then carried for 8 further passages in rabbits by using infected rabbit blood as transfer material. Aside from a febrile response, no other symptoms were observed in inoculated rabbits.

[12] Levaditi, C., Harvier, P., et Nicolau, S., *Annales de l'Institut Pasteur*, 1922, **36**, 107.

[13] Ch'en, W. K., PROC. SOC. EXP. BIOL. AND MED., 1933-34, **31**, 1252.

[14] Coffey, J. M., *Am. J. Pub. Health*, 1934, **24**, 473.

[15] Cox, H. R., *Science*, 1941, **94**, 399.

[16] Waddell, M. B., and Taylor, R. M., *Am. J. Trop. Med.*, 1945, **25**, 225.

[17] Edwards, J. T., Report of the Imperial Bacteriological Laboratory, Muktesar, India, for the 2 years ending March 1924, p. 32.

15541

Serial Passage of Hog Cholera Virus in Rabbits.

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Successful adaptation of rinderpest virus of cattle to the rabbit[1] naturally led to similar experiments with hog cholera virus since both of these agents are quite specific for their natural hosts. In the original work on rinderpest alternate transfer of virus from its natural host to the rabbit and then back to the calf was the procedure that made for success. With this alternating technic virulence of the rinderpest virus for the rabbit increased progressively and, following this increase, evidence of disease was found in the rabbit. The results of the experiments on hog cholera virus follow.

Virus Strains Used. Strain A was obtained through the courtesy of Dr. TenBroeck[2] and, as reported by him, it was one used for vaccination in the central portion of the United States. In his work this strain was grown in the presence of fresh minced swine testicle on the chorioallantoic membrane of embryonated eggs for 13 transfers, followed by an equal number of transfers on agar, making 26 transfers in all. From the last transfer lyophilized material was kept from April 5, 1941 until March 24, 1945 when it was injected into a pig. For further preservation spleen from this animal was kept frozen with

[1] Baker, J. A., *Am. J. Vet. Research*, 1946, **7**, 179.

[2] TenBroeck, C., *J. Exp. Med.*, 1941, **74**, 427.

typical temperature curve of a rabbit injected with the 8th rabbit passage spleen-virus is shown in Fig. 1.

Proof for Identity of the Virus. A pig injected with the 6th rabbit passage virus reacted with a 6-day febrile period but survived, and was subsequently placed in contact with 2 pigs which sickened, following injection with the original hog strain of Lederle blood virus. These animals were kept in an uncleaned pen with 2 normal contact control pigs. The 2 animals injected with the hog strain of the virus died and the 2 control swine showed fever after 5 and 6 days of contact respectively, and subsequently died on the 12th and 19th days. On autopsy, typical lesions of hog cholera were observed in the dead swine whereas the pig, injected with the 6th rabbit passage virus, remained asymptomatic during the entire 30-day observation period. On the 34th day this pig was injected with 2 ml of a 1:100 dilution of swine blood infected with hog cholera virus (Lederle stock strain). Normal swine injected simultaneously came down with typical symptoms of hog cholera whereas the pig which was previously injected with the 6th rabbit passage virus remained afebrile and symptom-free during the whole observation period.

Swine injected with the 8th and 9th rabbit passage spleen-virus (Table I), were autopsied after death and typical lesions of hog cholera were observed.

Propagation of the Virus in Rabbits by Injections of Infectious Blood. A pig injected with a spleen suspension of the 8th rabbit passage virus was bled when febrile on the 5th day after inoculation and 2 ml of its defibrinated blood were injected into the ear vein of a rabbit. The rabbit became febrile (105.4°F) 20 hours after inoculation at which time 20 ml of blood were taken by heart puncture. The blood was defibrinated and 2 ml were injected intravenously into each of 3 rabbits. Two of the animals became febrile and their blood was passed to another group of rabbits. By means of this technic, the virus has been propagated for 8 passages in rabbits. The data are summarized in Fig. 2. It may be observed that not all rabbits inoculated with the infected blood reacted with fever. However, in the later passages more animals showed a febrile reaction, and the temperature peaks reached higher levels and persisted for longer periods than in those rabbits inoculated with the spleen-passage virus, although in the latter case it was difficult to ascertain how long the febrile period lasted because the animals were sacrificed immediately after the supposed peaks of fever were reached.

In Fig. 3 are shown temperature curves of rabbits inoculated respectively with the 6th and 7th rabbit blood-passage virus. It may be observed that the temperature of the rabbit inoculated with the 6th passage virus started to rise between the 68th and 70th hour after inoculation, reached its peak on the 74th hour when the animal was bled and remained above normal for 16 hours. Blood of this rabbit produced a typical clinical picture of hog cholera in an inoculated pig which died on the 13th day after inoculation. On the other hand, the temperature curve of the rabbit injected with the 7th rabbit blood passage virus followed a slightly different course. A temperature of 105.0° was reached on the 50th hour after inoculation when the animal was bled. Then the temperature curve dropped rather suddenly and remained at a 103.2° level during 2 hours, returning back to a 105.4° level at the 56th hour after inoculation. During the next 26 hours the temperature was either at a subfebrile (103.8°) level or at a febrile level (105.8°), returning back to normal at the 94th hour after inoculation and remaining normal until the end of the observation period. Blood of this rabbit injected into a pig again produced a clinical picture of hog cholera. Up to now, no symptoms other than a febrile response were observed in rabbits injected with the rabbit blood-passage virus.

Discussion. The data presented above indicate that hog cholera virus has been successfully passed through several generations in rabbits by the use of an alternating passage method. However, it was apparently also essential to take temperatures of the rabbits frequently day and night during the entire observation period—and to make pas-

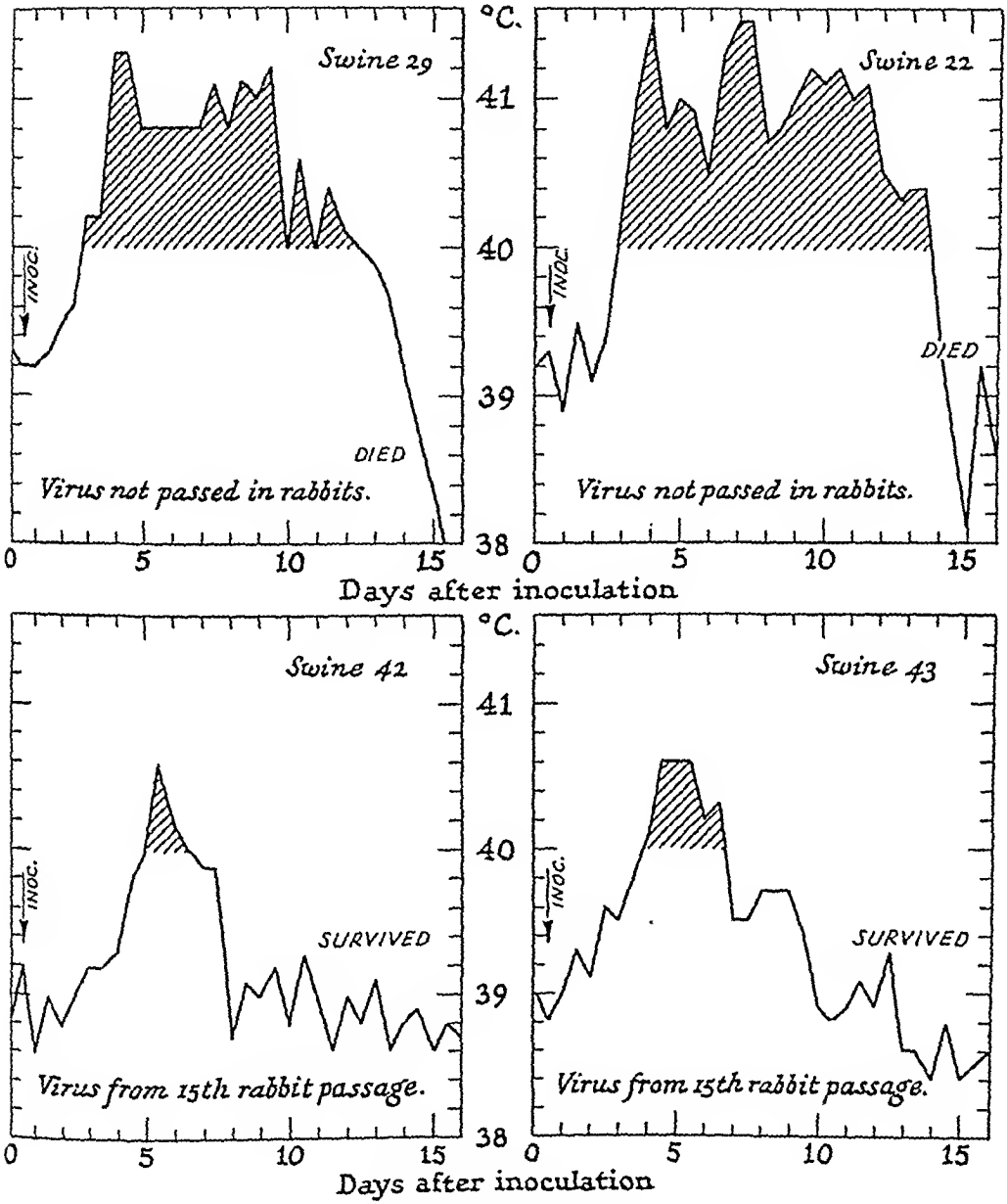


FIG. 1.

Thermal reaction in pigs given hog cholera virus (strain A) not transferred in rabbits and from the 15th serial passage.

Strain B. In the first passage 0.1 g of the rabbit spleen infected pigs but 0.01 g did not. Transfer to a second rabbit failed, since 0.1 g of this spleen did not infect a pig. The alternating technic described briefly above and more fully in the rinderpest paper[1] was then attempted. As Table II shows, swine were infected with 0.1 but not with 0.01 g of spleen from rabbits in the first 3 alternating passages. In no case was the virus detected in the 2nd consecutive rabbit passage. After the 4th and 5th alternation, 0.01 g of

TABLE I.
Effect on Swine of Hog Cholera Virus (Strain A) Before and After Serial Passage in the Rabbit.

No. of serial transfers in rabbits	Swine		Results in swine				
	No.	Wt, lb	Incubation period, days	Duration of fever, days	Highest temp., °C	Outcome of illness	Reaction to injection of virulent virus
0	1	37	3	9	41.0	Died	
0	22	27	3	11	41.5	"	
0	29	30	3	10	41.3	"	
0	37	24	3	11	41.8	"	
0	40	21	3	8	41.5	"	
5 (a)*	8	80	3		41.5	Killed	
5 (b)	11	70	3		41.2	"	
10 (b)	13	70	?	0	39.3	Survived	Immune
10 (b)	20	25	?	0	39.7	"	"
10 (a)	27	30	3	4	41.3	"	"
15 (b)	42	32	5	2	40.6	"	"
15 (a)	43	30	4	3	40.6	"	"

* (a) indicates first group of serial passages; (b) second group of serial passages.

dry ice until January 2, 1946 when this work began.

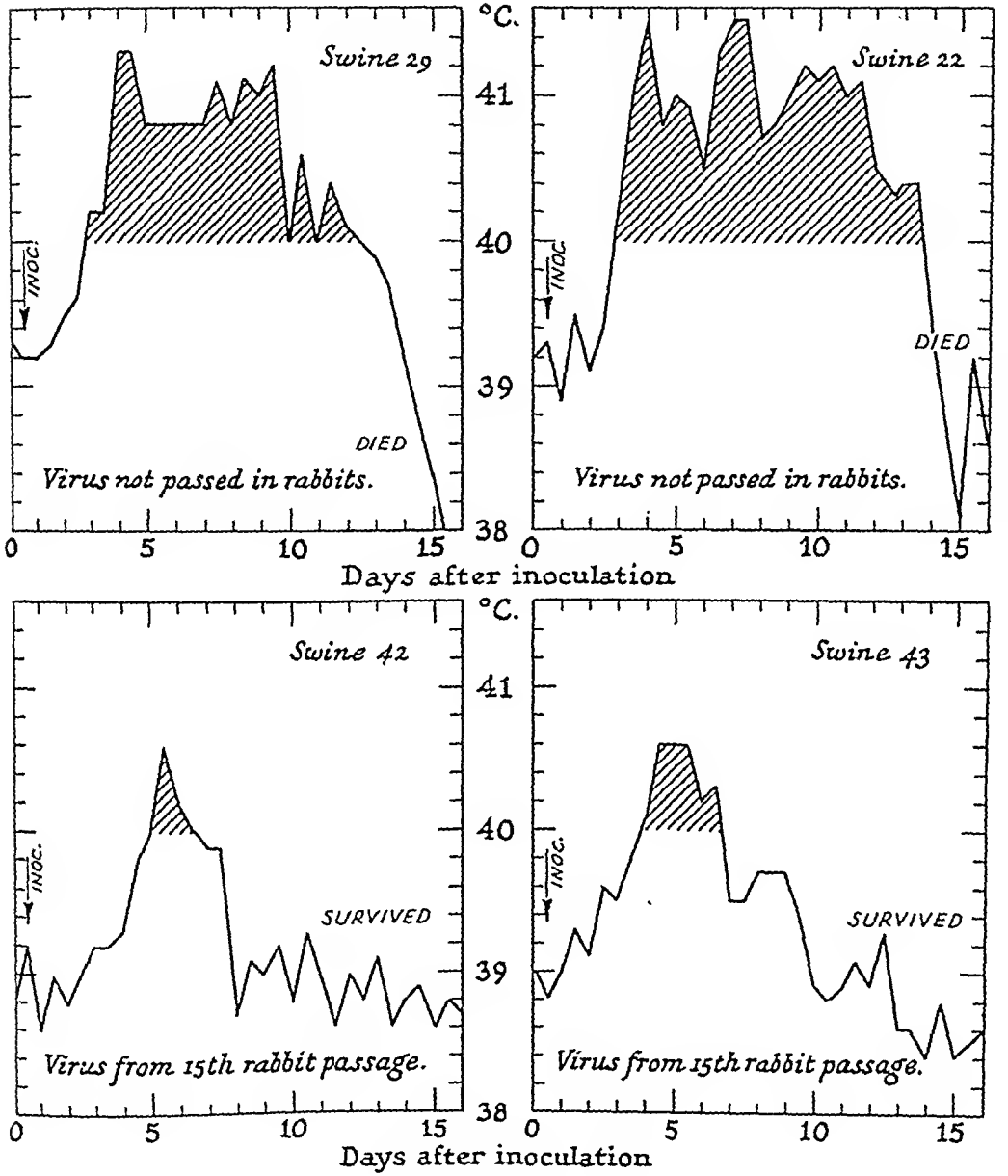
Strain B was obtained through the courtesy of Dr. Shope. This strain was recovered by him from a natural outbreak May 26, 1944 and was subsequently carried through 5 transfers in swine. In interim periods between transfers the virus was kept as infected blood in a refrigerator.

Rabbits Used. The rabbits used were from mongrel stock, largely Polish-Dutch, from 3 to 5 months old weighing 1500 to 2500 g.

Procedure Used. Each of 2 rabbits was inoculated intravenously with 1 cc of a 10% suspension of spleen obtained from an infected pig at the height of illness. The rabbits were killed after 5 days and a portion of spleen from each was used for making another 10% suspension. Two rabbits were given 1 cc each of this suspension and injections of the same suspension were made into swine to determine the amount of virus present. Spleen tissue was also frozen in CO_2 and was available for retest if necessary. Tests for contaminating organisms made on blood agar slants and sealed meat medium showed occasional cocci from a few of the pig spleens. Suspensions of rabbit spleens showed no growth. Temperatures were taken and observations made on the rabbits and swine in the morning and evening. Swine that died were autopsied and the diagnosis was made on the finding of typical lesions. Any animals that survived were reinoculated with at least 100 times the infective dose and a control animal injected with the same virus was kept in the same room until its death. If the surviving animal showed no signs of illness from this test injection and exposure period, it was evident that hog cholera virus had been present in the material used for the first inoculation.

Results. Strain A. Test of the spleen suspension of the first rabbit passage showed that 0.01 g of tissue would infect swine, whereas 0.001 g would not. The spleen suspension of the 2nd and subsequent rabbit passages was not titrated, but 0.1 g contained virus, as shown by swine inoculation on the 2nd, 5th, 10th, and 15th passages. These results were repeated in a similar series using as starting material spleen from a different pig and the composite results are shown in Table I.

As Table I shows, strain A was twice passed serially through rabbits for 15 transfers. There was noted a decrease in virulence for swine, as rabbit spleen suspensions from the 10th and 15th serial passages injected intramuscularly produced fever of short duration as the only sign of illness (Table I and Fig. 1). This was followed by complete immunity to an intramuscular injection of 100 times the amount of fully virulent hog cholera virus that produced death in a control animal kept in the same room. Virus attenuated in this way by serial passage in rabbits may make a practical and inexpensive vaccine.



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Strain B. In the first passage 0.1 g of the rabbit spleen infected pigs but 0.01 g did not. Transfer to a second rabbit failed, since 0.1 g of this spleen did not infect a pig. The alternating technic described briefly above and more fully in the rinderpest paper[1] was then attempted. As Table II shows, swine were infected with 0.1 but not with 0.01 g of spleen from rabbits in the first 3 alternating passages. In no case was the virus detected in the 2nd consecutive rabbit passage. After the 4th and 5th alternation, 0.01 g of

TABLE II.
Hog Cholera Virus (Strain B) Alternated Between Swine and Rabbits.

Alternating passage in swine	Serial passage in rabbits	Test of rabbit spleen for virus by swine injection	
		Amt (g)	Result
1st	1	0.1	+*
		0.01	—
	2	0.1	—
2nd	1	0.1	+
		0.01	—
	2	0.1	—
3rd	1	0.1	+
		0.01	—
	2	0.1	—
4th	1	0.01	+
		0.001	—
	2	0.1	—
5th	1	0.01	+
		0.001	—
	2	0.1	—
6th	1	0.001	+
		0.0001	—
	2	0.1	+
	3	0.1	—
7th	1	0.001	+
		0.0001	—
	2	†	
	3	0.1	—
8th	1	0.001	+
		0.0001	—
	2	†	
	3	0.1	—

* + indicates virus present; —, no virus.
† Not tested.

spleen then infected swine. Again no virus was detected in the 2nd consecutive rabbit passage. After the 6th, 7th, and 8th alternation, 0.001 g then infected swine. With this last increase of virus in the rabbit, transfer was successful for 2 but not 3 serial rabbit passages.

Swine inoculated with infective spleen suspensions from rabbits in the 1st, 2nd, and 3rd alternating passages showed an incubation period of 3 days while a time interval of 4 to 7 days between injections and appearance of fever was noted in those given a similar amount of virus from subsequent alternating passages. It seems evident, therefore, that adaptation of strain B for rabbits was caused by alternating the virus between swine and rabbits.

Effect of Hog Cholera Virus on Rabbits. None of the rabbits inoculated with either strain A or B showed any temperature elevation in early passages. In later passages a slightly increased temperature was noted in some animals inoculated with either strain but temperatures exceeding 40°C seldom occurred. No other signs of illness were noted. None of the animals upon autopsy showed lesions referable to hog cholera.

Discussion. A review by Muir[3] of the divergent results obtained by others in transfers of hog cholera virus to animals other than swine would suggest that some strains are adaptable while others are not. The complete adaptation to rabbits of strain A

[3] Muir, R. O., *J. Comp. Path. and Therap.*, 1940-1943, 53, 237.

and incomplete adaptation of strain B in our work would support this idea. Also, adaptation of yellow fever by Theiler and Smith,[4] dengue by Sabin and Schlesinger,[5] and perhaps other viruses to animals has not been obtained regularly.

The fact that strain A had been kept under laboratory conditions for a long period and had been cultured outside the body of swine may have influenced its adaptability to the rabbit, although it is possible that strain A was naturally more adaptable. On the other hand, strain B had been isolated recently. It showed poor adaptability and required 6 alternating passages before virus increase occurred in the rabbit spleens and it could be transferred consecutively in the rabbit.

Since neither strain A nor B produces definite signs of illness in rabbits, a study of other strains and perhaps in other laboratory animals should be made especially by the alternating method which, as was shown with strain B and rinderpest, allows opportunity for development of any latent pathogenic potentiality. Such a study may give results similar to those obtained with rinderpest and supply the laboratory animal that has been needed in hog cholera research.

Summary. Two strains of hog cholera virus were inoculated into rabbits. One strain (A) continued in serial passage, became attenuated for swine and, after 15 rabbit passages, produced a febrile reaction of short duration as the only sign of illness. This attenuated strain fully immunized swine to the virulent hog cholera virus. The other strain (B) was present in rabbits for 1 but not 2 consecutive passages. With 6 continued transfers alternately between swine and rabbits, the amount of virus in the rabbit's spleen attained 100 times that found in the initial rabbit transfer. Serial passage in the rabbit was then successful for 2 but not 3 transfers. Evidence of attenuation of this strain was the lengthened incubation period in swine inoculated with the rabbit-passed material. Possible explanations for the difference in adaptability of these strains are discussed.

[4] Theiler, M., and Smith, H. H., *J. Exp. Med.*, 1937, **65**, 767.

[5] Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, **101**, 640.

15542

Antihistaminic Substances in Histamine Poisoning and Anaphylaxis of Mice.

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The conditions under which mice can be sensitized and the manifestations of sensitization in mice closely simulate those of anaphylaxis in guinea pigs and other animals (Braun,[1] Schultz and Jordan,[2] Ritz,[3] Sarnowski,[4] Bourden,[5] Weiser, Golub and Hamre[6]). Active sensitizations can be produced with various protein substances, horse, sheep, cow, guinea pig serum, or egg white; passive sensitizations with immune rabbit serum, antihorse guinea pig serum, antihorse rabbit serum, antipneumococcus Type I rabbit serum, etc. The sensitizations are specific and their duration varies from several weeks to several months. Refractoriness occurs after recovery from shock and active desensitization is obtained by

[1] Braun, H., *Munch. med. Wschr.*, 1909, **37**, 1880; *Z. Immunf.*, 1910, **4**, 590.

[2] Schultz, W. H., and Jordan, H. E., *J. Pharm. and Exp. Therap.*, 1911, **2**, 375.

[3] Ritz, H., *Z. Immunf.*, 1911, **9**, 321.

[4] Sarnowski, V., *Z. Immunf.*, 1913, **17**, 577.

[5] Bourdon, K. L., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 340.

[6] Weiser, R. S., Golub, O. J., and Hamre, D. M., *J. Inf. Dis.*, 1941, **68**, 97.

the usual desensitization methods. Specific precipitins are formed in mice actively sensitized against egg white with a titer varying from 1:100 to 1:400.

From all these facts it has been concluded that the "protein shock" in mice is a true anaphylaxis; they fulfill indeed Doerr's[7] criteria of allergy.

The mechanism which ultimately leads to the various anaphylactic manifestations in the different animal species is certainly not the same. The tissue which becomes the principal site of sensitization in guinea pigs is that of the lungs; in rabbits, that of the vascular system; in dogs, that of the liver; etc. The site of sensitization in mice is unknown.

The similarity of the symptoms of anaphylactic shock in guinea pigs or dogs to those of histamine poisoning was first recognized by Dale and Laidlaw.[8] The significant increases of the histamine level in the blood during anaphylactic shock in guinea pigs and dogs are strong support for the "histamine theory" of anaphylaxis. It is today commonly accepted that at least in these animal species the antigen-antibody reaction leads to a liberation of histamine or a histamine-like substance, which is the ultimate cause of the anaphylactic shock. These findings have been interpreted in a more general way, and histamine is considered to be the principal offender in all cases where antigen-antibody reactions in allergy lead to anaphylactic symptoms or to reactions of a similar nature.

However, objections have been raised against such a generalization. For instance, unlike anaphylaxis in guinea pigs and dogs, the histamine blood level decreases in anaphylaxis of rabbits, horses and calves (Code and Hester,[9] Rose and Weil[10]). The connection between allergic manifestations and histamine is especially debatable in mouse anaphylaxis. Whereas the very small dose of 0.6-0.8 mg per kg body weight of intravenously-injected histamine phosphate kills guinea pigs almost instantly, mice are very resistant to histamine, and many animals survive the intravenous injection of 500 mg per kg body weight. In our own experiments, the dose of 750 mg per kg body weight was lethal in all cases. According to these figures, the mouse is about 1,000 times more resistant to histamine than the guinea pig.

The histamine content of the normal mouse averages about 10 mg of histamine per kg body weight. Since mice present the first signs of histamine shock only when 100 mg or more of free base are injected, it is rather improbable that histamine is involved in anaphylactic shock in mice.

In an endeavor to study this question a new approach was used, which was made possible by the recent introduction of the so-called antihistaminic substances supposed to counteract histamine specifically, and considered by many investigators as biologic reagents for histamine. It was of interest to investigate whether histamine poisoning in mice and mouse anaphylaxis responded to these antihistaminic substances in the same way as anaphylaxis of the guinea pig and the dog.

Experimental. I. Influence of Antihistaminic Substances Upon Histamine Poisoning in Mice. Since the toxicity of histamine for mice depends largely upon the concentration of histamine and the rapidity of injection, 2% solutions of histamine phosphate were uniformly used in all experiments; the solutions were injected at the rate of 1 ml per minute. Under these conditions 500 mg of histamine phosphate per kg body weight killed 50% of the mice; 375 mg per kg, 44%. 250 mg per kg body weight occasionally produced a brief period of muscular incoordination and convulsions, but after a few minutes almost all animals regained their normal state.

If antihistaminic substances such as Pyribenzamine* or Benadryl† were given in subcutaneous injections of 10 or 25 mg per kg body weight 15 minutes before the histamine injection, the toxicity of histamine was not

[7] Doerr, R., *Arch. f. Dermat.*, 1926, **151**, 3.

[8] Dale, H., and Laidlaw, P., *J. Physiol.*, 1911, **43**, 182.

[9] Code, C. F., and Hester, H. R., *J. Physiol.*, 1939, **127**, 71.

[10] Rose, B., Braun, H., and Weil, P., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 494.

* Trade name for N'-pyridyl-N'-benzyl-N-dimethyl ethylene diamine monohydrochloride (Ciba).

† Trade name for dimethyl amino ethyl benzhydryl ether hydrochloride (Parke, Davis & Co.).

TABLE I.
Toxicity for Mice of Histamine Phosphate Alone and in Combination with "Antihistaminic Substances."

Histamine doses mg/kg i.v.	No antihistaminic substance	Pyribenzamine		Benadryl	
		10 mg/kg	25 mg/kg	10 mg/kg	25 mg/kg
750	10/10*	3/3		6/6	
500	10/20	11/11	11/11	10/12	14/14
375	10/23	9/11	16/16	11/12	10/10
250	1/13	3/8	11/15	0/5	0/5

Histamine phosphate, 2% solutions, intravenous injection, 1 ml per minute.
Pyribenzamine and Benadryl given 15 minutes before histamine by subcutaneous injection.
* Denotes animals dead/animals tested.

decreased, as is usually the case with guinea pigs or dogs, but, on the contrary, was strongly enhanced. Histamine phosphate alone in a dose of 375 mg per kg body weight was lethal for 10 out of 23 mice (43%). In combination with 25 mg per kg Pyribenzamine, the same dose of 375 mg of histamine killed 16 out of 16 mice (100%) and in combination with 25 mg per kg Benadryl, 10 out of 10 mice (100%). A similar significant increase in toxicity occurred with a combination of 500 mg per kg histamine phosphate with 25 mg per kg Pyribenzamine or Benadryl (from 50% mortality in controls, to 100% with either substance). The dose of 25 mg per kg Pyribenzamine, or Benadryl is nontoxic for mice.

The results (shown in Table I) indicate that the increase in toxicity of histamine is proportional to the dose of Pyribenzamine or Benadryl previously injected. These surprising results indicate that Pyribenzamine or Benadryl, which strongly and specifically counteract and neutralize histamine in guinea pigs and dogs, are definite synergists of histamine in mice.

II. *Influence of Pyribenzamine Upon Active Anaphylaxis in Mice.* The picture of a fully developed mouse anaphylaxis is the following: Five to 10 minutes after the shocking dose is injected, the animals show some excitement, but rapidly become quiet. After a short time, increasing difficulty in breathing develops. This state is followed by loss of coordination, and about 20 minutes later a progressive paralysis of the body starts, beginning with the hind legs. In those instances in which the animal dies, this paralytic state is soon succeeded by a short period of violent convulsions and coma, in which the animals succumb in about half an hour. The entire sequence is sometimes over in 15 minutes, but may last an hour or more. The symptoms are quite similar to those seen in anaphylaxis in guinea pigs, rabbits, or dogs—they merely extend over a much longer period of time.

210 mice were sensitized, in 2 separate groups, by 4 consecutive intraperitoneal injections of 1 ml each of undiluted horse serum every other day. Twenty-one days later a challenging dose of 1 ml of undiluted horse serum, warmed to 37°C, was injected intravenously into 169 mice still alive at that time. Preliminary experiments had shown that this amount, representing in sensitized mice about 2 shocking doses, did not affect normal mice.

Fifty-eight out of the 169 surviving sensitized mice served as controls and received the challenging injection without any other treatment. All of them went into severe shock with a mortality of 89%. The other 111 mice were divided into 4 groups of 20 to 30 animals each and received subcutaneous injections of Pyribenzamine in doses of 10, 25, 30 or 50 mg per kg body weight respectively, 15 minutes before the challenging dose of horse serum was injected intravenously. The challenging injection of horse serum produced in animals, without exception, the depressive stage of shock with varying degrees of prostration. Nevertheless, about one-half of the mice treated with 10 to 30 mg of Pyribenzamine remained free from convulsions and recovered. Thus, the morbidity picture, compared to the controls, was somewhat improved and the mortality rate definitely

TABLE II.
Effect of Pyribenzamine upon Mouse Anaphylaxis.

	Controls No treatment	Treatment with Pyribenzamine (mg/kg) 10	25	30	50
No. of animals dead in shock over No. of animals used	53/58	18/29	13/31	12/24	19/27
% of *deaths in shock*	89%	65%	42%	50%	70%

decreased. In contrast to a mortality rate of 89% for the controls, only 65% of the mice pretreated with 10 mg of Pyribenzamine per kg body weight died (18 out of 29); 42% of those pretreated with 25 mg of Pyribenzamine (13 out of 31); 50% after pretreatment with 30 mg of Pyribenzamine (12 out of 24); and 70% after pretreatment with 50 mg of Pyribenzamine. This last dose of Pyribenzamine is definitely toxic for mice and it is probable that a number of the deaths included in the 70% are due to Pyribenzamine. (LD_{50} of Pyribenzamine by subcutaneous injection for mice is 75 mg per kg body weight). Table II shows the results obtained.

It is significant that in no case was complete freedom of all shock symptoms ever obtained, even with high doses of Pyribenzamine, although significant protection against convulsions and death was afforded. In any event, protection conferred on the sensitized mice was much less spectacular, as compared to the certainly more complete protection obtained in sensitized guinea pigs with doses of Pyribenzamine only 1/50 as great.

Discussion. It has often been suggested that a positive correlation exists between a species sensitivity to histamine and the ease with which one may induce anaphylaxis in this species. Indeed, guinea pigs, dogs, rabbits, or man which are highly sensitive to histamine, become sensitized quite easily. In these same species, Pyribenzamine and other antihistaminic substances have been shown to be most powerful antagonists of histamine, as well as of anaphylaxis (Dekanski,[11] Mayer, Huttrer and Scholz,[12] Mayer,[13] Yonkman, Hays and Rennick,[14] Arbesman, Koepf and Miller[15]).

In vitro, small doses of Pyribenzamine prevent several typical histamine effects on isolated guinea pig intestines, uterus, or lungs; *in vivo,* 5 mg per kg protect guinea pigs against 100 and more lethal doses of histamine. Anaphylactic shock in guinea pigs can be prevented by doses as small as 0.1 to 0.5 mg per kg body weight; somewhat higher doses are active against histamine poisoning or anaphylactic shock of dogs (3-3.5 mg per kg body weight).

Contrary to guinea pigs and dogs, the mouse is highly resistant to histamine; it tolerates about 1,000 times more histamine than the guinea pig. Correspondingly, the mouse is very resistant to any sensitizing procedures.

A similar difference existed in the response to antihistaminic substances. Pyribenzamine and Benadryl not only failed to protect mice from histamine poisoning, but definitely acted as synergists to histamine. After an injection of 25 mg per kg body weight of Pyribenzamine, for instance, the toxicity of histamine in mice was doubled.

That the antihistaminic substances act quite differently in mice than they do in guinea pigs seems not to be a fortuitous occurrence, but rather seems to be in agreement with the theory that antihistaminic substances are competitors of histamine.

An analysis of the general and quite uncharacteristic picture of [illegible] histamine poisoning in mice does [illegible] ny answer to the question of whethe[illegible] mice in the same manne[illegible] same organs and by ider[illegible] in guinea pigs and dogs[illegible] protection by antihistan[illegible]

11 Dekanski, J., *J. Physiol.*, 1945, **104**, 151.

12 Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science*, 1945, **102**, 93; *Fed. Proc.*, 1945, **4**, 129.

13 Mayer, R. L., *J. Allergy*, 1946, **17**, 153.

14 Yonkman, F. F., Hays, H. W., and Rennick, B., *Fed. Proc.*, 1945, **4**, 144.

15 Arbesman, C. E., Koepf, G. F., and Miller, G. E., *J. Allergy*, 1946, **17**, 203.

case of histamine poisoning of mice seems to indicate that different mechanisms are involved. On the other hand, the synergism of histamine and "antihistaminic" substances cannot be explained by a possible histamine-like activity of these "antihistaminic substances." There is no such action in mice. We rather conclude that in the mouse, histamine affects organ systems other than those affected in histamine-sensitive animals, and that in these points of attack, antihistaminic substances are unable to compete with histamine. On the contrary, each poison acts on its own, both toxicities being additive in effect.

It seems quite inconsistent with the synergistic action of histamine and Pyribenzamine that the latter should have a certain protective action in mouse anaphylaxis. The very fact that Pyribenzamine in the mouse does not act as an antihistaminic agent, leads to the belief that this "protection" against mice anaphylaxis is not due to a specific antianaphylactic effect, comparable to that which Pyribenzamine exercises in anaphylaxis of guinea pigs or dogs. It is rather probable that some pharmacologic properties of Pyribenzamine other than its antihistaminic power are responsible for this protection. It may be that it acts here through its local anesthetic power, since it is known that general anesthetics, as well as local anesthetics, have a definite influence upon anaphylaxis (Wolfsohn[16]). The same problem has been discussed in a study concerning the influence of Pyribenzamine upon contact dermatitis in guinea pigs (Mayer[17]). Other experiments by Yonkman, Hays, Chess and Rennick[18] seem to indicate that the general pharmacological activity of an antihistaminic agent such as Pyribenzamine may well include other properties than those associated with antihistaminic power.

Further experiments are necessary to correlate allergy in mice with that of other animals and to identify the "anaphylactic poison" of mouse anaphylaxis, which quite probably is not histamine.

Conclusions. (1) "Antihistaminic substances" act as synergists in the histamine poisoning of the mouse; (2) contrary to the action on histamine poisoning, a certain protective power in mouse anaphylaxis has been observed; (3) the possible conclusions following from these observations are discussed.

16 Wolfsohn, G., *The Palestine and Near East Med. J.*, 1944, 3, 11.

17 Mayer, R. L., *Ann. Allergy*, in press.

18 Yonkman, F. F., Hays, H. W., Chess, D., and Rennick, B., *J. Pharm. and Exp. Therap.*, in press.

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Concentration and Properties of the Adrenocorticotrophic Substance in Female Human Urine.

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Adrenocorticotrophin has been demonstrated to be present in the blood serum[1] and in the anterior pituitary gland. Its possible presence in the urine has been suggested by the effects of injecting urine from pregnant[2] and nonpregnant[3] women on the adrenal cortex of young guinea pigs. No attempt has been made to identify the substance which may have caused the adrenal hyperplasia. Since the corpus luteum hormone[4] and estrogens[4,5] can induce hyperplasia of the

1 Golla, M. L., and Reiss, M., *J. Endocrinology*, 1942, 3, 5.

2 de Boissezon, P., *Bull. histol. appliq. physiol.*, 1936, 13, 129.

3 Blumenthal, H. T., *J. Lab. Clin. Med.*, 1945, 30, 428.

4 Janes, R. G., and Nelson, W. O., *Am. J. Physiol.*, 1942, 136, 136.

5 Cutilla, U. Y., *Endocrinology*, 1940, 26, 123.

TABLE I.
The Effect of Female Urine and Fractions Thereof on the Hypertrophy of Young Male Rat Adrenals.

Exp. No.	No. of rats	Starting wt of rats g	Material tested	Adrenal wt, mg	Mg adrenal per 100 g body wt
I	12	50.9	*Fresh whole female urine*—0.5 cc intraperitoneally, twice daily for 4 days	19.5	36.1
	5	49.6	*Dialysate of above urine*—Administered as above	19.8	35.6
	12	50.8	*Control*—isotonic saline as above	11.2	17.8
II	7	40.7	*Conc. whole urine* (2.5:1)—0.5 cc intraperitoneally, twice daily for 4 days	12.6	24.5
	7	40.7	*Acetone ppt. of dialyzed urine* (Prep. W-1)—6 mg daily, administered as above	14.6	29.3
	7	40.9	*Control*—isotonic saline (pH 7.7)	8.0	15.4
III	7	37.3	*Conc. whole urine*—0.5 cc intraperitoneally, twice daily for 4 days	10.8	27.1
	7	39.0	*Acetone ppt. of dialyzed urine* (Prep. W-2)—6 mg daily, administered as above	14.6	31.1
	6	38.3	*Control*—isotonic saline (pH 7.7)	8.4	17.3

adrenal cortex, the possibility that the cortical hyperplasia might be due to the sex hormones found in the urine has not been eliminated.

In this paper, we present evidence that the adrenocorticotrophic activity of human urine does not depend on the ovarian hormones and that it is probably due to a protein which may be the excretory form of pituitary adrenocorticotrophin.

Experimental. Freshly voided human female urine, dialyzed urine and precipitated urinary proteins were tested for adrenocorticotrophic activity in white rats by the methods of Moon[6] and Sayers, *et al.*[7,8] for pituitary adrenocorticotrophins.

Young male rats (23-30 days old) were injected intraperitoneally twice daily for 4 days, with 0.5 cc portions of urine which had been voided and filtered within the previous hour. The urine was obtained by pooling the output of 5 nonpregnant nonmenstruating young women. The control rats received isotonic saline solution at the same time. After the experimental period, the rats were sacrificed and the adrenals removed and weighed. The results of this experiment (I) are shown in Table I. The greater adrenal weight of the experimental rats over that of the controls indicates the presence of adrenocorticotrophic activity in the urine, confirming previous observations.[3]

A sample of urine was dialyzed for 24 hours at 0° to remove the greatest portion of the estrogens present. When the dialyzed urine was tested as in the above experiment, similar results were obtained (Table I, Exp. I). This experiment indicates that the urinary estrogens are not the causative agents for the adrenal hypertrophy.

In order to have a standard supply of urinary adrenocorticotrophin on hand, a concentrate of female urine was made in the following manner. Freshly voided urine was filtered and dialyzed with agitation against 20 volumes of distilled water at 0° for 24 hours. The dialysis was repeated with another 20 volumes of fresh distilled water. The dialyzed urine was then concentrated to approximately ⅕ volume by pervaporation under toluene. To the concentrated urine 5-6 volumes of acetone were added and the mixture kept in the cold room for 48 hours. The precipitate was centrifuged, washed twice with acetone and dried over P_2O_5. A pooled sample of 2.5 l. of urine yielded 505 mg of a light gray powder which gave a strong biuret test and contained 7.34% nitrogen (microkjeldahl, Prep. W-1). When another 1 liter sample of urine was put through this

6 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 649.

7 Sayers, G. M., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1946, **38**, 1.

8 Sayer, M., and Sayers, G., *Proc. Fed. Am. Soc. Exp. Biol.*, 1946, **5**, 200.

TABLE II.
Effect of Female Urine and Fractions Thereof on Adrenal Ascorbic Acid of Young Male Rats.

No. of rats	Rat wt, g	Material tested	Ascorbic acid per adrenal μg	Mg ascorbic acid per 100 g adrenal
5	40.2	Fresh whole urine—2.0 cc	16.9	277
5	44.6	Acetone ppt. of dialyzed urine (Prep. W-1); 6 mg in 2.0 cc isotonic saline-phosphate buffer (pH 8.0)	15.5	267
5	36.4	Prep. W-1 heated at 100° for 30 min; 6 mg in 2.0 cc isotonic saline-phosphate buffer (pH 8.0)	45.1	490
4	35.0	Crystalline egg albumin—6 mg in 2.0 cc isotonic-saline-phosphate buffer (pH 8.0)	32.6	473
14	39.5	Control—4 mg gelatin in 2.0 cc isotonic saline-phosphate buffer (pH 8.0)	32.6	501

procedure, a yield of only 52 mg of powder was obtained (Prep. W-2).

A solution of each of the above preparations in isotonic saline-0.01 M phosphate buffer (pH = 7.7; 4 mg per cc) was tested in the manner previously described. The results (Table I, Exp. II and III) show that both preparations have adrenocorticotrophic activity. These experiments are a further demonstration that the urinary steroid hormones do not cause the observed adrenal hypertrophy. Dialysis and the acetone treatment can be expected to remove all but traces of urinary steroids.

The method of Sayers, *et al.*[7,8] depends on the fact that pituitary adrenocorticotrophin will cause a rapid and profound decrease in the ascorbic acid level of the adrenals. The method was modified in the following manner to test for the presence of adrenocorticotrophin in urine. Young male rats were anesthetized with nembutal and 2.0 cc of the solution to be tested were injected intraperitoneally. After 1.5 hours, the animals were sacrificed, the adrenals removed and the ascorbic acid content determined. The ascorbic acid was measured by the method of Carruthers.[9] In our hands, this method was found satisfactory in that known amounts of ascorbic acid could be estimated with an error of less than 7%.

Fresh female urine and Prep. W-1, when tested in the above manner, showed a marked effect in depressing the adrenal ascorbic acid as compared to the controls (Table II). The control animals received 4 mg of gelatin in 2 cc of isotonic saline-phosphate buffer (pH = 8.0). The gelatin was used as a foreign-protein control. When a solution of Prep. W-1 was heated in a water-bath at 100° for 30 minutes, it no longer possessed any depressing effects on the adrenal ascorbic acid level. Since Prep. W-1 is also nondialyzable, this would seem to indicate that the active material is protein in nature. That nonspecific antigenic proteins are probably ineffective in this test was shown when egg-albumin (3X recrystallized) was assayed and found to have no effect on the adrenal ascorbic acid level.

A solution of Prep. W-1 was also tested on hypophysectomized rats. One of the adrenals of the hypophysectomized animals was removed and a solution of 6 mg of Prep. W-1 in isotonic saline-phosphate buffer was injected. After 1.5 hours the second adrenal was removed and the ascorbic acid level in each determined. A depression in adrenal ascorbic acid (145 mg/100 g adrenal tissue) was found. This indicates that the urinary adrenocorticotrophin acts in the same manner as the pituitary adrenocorticotrophins in that both decrease the ascorbic acid level of the adrenals markedly in a relatively short time. Further, the urinary adrenocorticotrophin acts without the mediation of the pituitary.

It can, therefore, be seen that the urinary and pituitary adrenocorticotrophins are qualitatively similar in their effects. This establishes the possibility that they are identical, or, at least, closely related, and that the urinary adrenocorticotrophin is an excretory form of the pituitary adrenocorticotrophin.

[9] Carruthers, A., *Ind. Eng. Chem., Anal. Ed.*, 1942, 14, 826.

The determination of the presence of adrenocorticotrophin in male urine and the identification of the adrenocorticotrophic protein in female urine are now under investigation.

Summary. Normal female urine contains adrenocorticotrophic activity which has been shown not to be due to its estrogen content. Being nondialyzable and thermolabile, the active material appears to be a protein. It is similar to pituitary adrenocorticotrophin in its ability to produce adrenal weight increases and to depress the adrenal ascorbic acid level in normal and hypophysectomized male rats.

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Effect of Boric Acid on Avian Malaria.

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The recent paper by Hardcastle and Foster[1] reporting encouraging results with the use of borax in the control of cecal coccidiosis in poultry suggested the possibility that boron derivatives might be effective as chemotherapeutic agents in certain types of avian malaria.

Trophozoite-induced *Plasmodium gallinaceum* infections were established in S.C. White Leghorn chicks weighing 50 g by intravenous inoculation of 200,000,000 parasitized erythrocytes per kg; trophozoite-induced *P. cathemerium* infections were induced in Peking ducklings weighing 50 g by the intravenous injection of 500,000,000 parasitized erythrocytes per kg. Sporozoite-induced *P. gallinaceum* infections were used in prophylactic tests and were established in chicks of 50 g weight by injecting intravenously 0.2 cc per chick of a suspension of sporozoites prepared by grinding 100 infected mosquitoes in 20 cc of chicken plasma.

[1] Hardcastle, A. B., and Foster, A. O., *Proc. Helminthological Soc. of Washington*, 1944, **11**, 60.

TABLE I.
Effect of Boric Acid on Trophozoite and Sporozoite Induced Avian Malaria Infections.

No. birds	Drug	Dose	Average % erythrocytes parasitized at peak of infection
	I. Trophozoite-induced *P. cathemerium* infections in ducklings.		
4	Boric acid	2.0% in diet	1.3
4	" "	1.0 " " "	6.6
3	Quinine	80 mg/kg p.o.	5.6
3	"	40 " "	3.1
6	Controls	—	25.6
	II. Trophozoite-induced *P. gallinaceum* infections in chicks.		
5	Boric acid	2.0 % in diet	2.4
5	" "	1.0 " " "	31.9
3	Sulfadiazine	0.05 " " "	<0.1
3	"	0.025 " " "	0.5
6	Controls	—	66.1
	III. Sporozoite-induced *P. gallinaceum* infections in chicks.		
4	Boric acid	2.0% in diet	0.01*
4	" "	1.0 " " "	1.7
4	Sulfadiazine	0.1 " " "	0.00
4	Controls	—	30.0
4	Quinine	0.4 " " "	22.0
4	Atabrine	0.4 " " "	19.7
4	Sulfadiazine	0.2 " " "	0.00
8	Controls	—	17.6

* One chick died, two showed zero counts.

The boric acid was administered by incorporating it in the diet, a commercial chick starting mash, in the amounts shown in Table I. The birds were given the drug-containing diet immediately after inoculation and were permitted to feed *ad libitum* throughout the period of the test. The trophozoite-infected birds were continued on the drug-diet for 5 days. The chicks receiving the sporozoite inoculation were fed the drug-diet for 3 days and were then transferred to the regular untreated stock diet for an additional 6 days.

At the end of the respective test period, thin blood smears were prepared, stained with Giemsa, and the effect of the therapy was judged by determining the number of parasitized cells among 10,000 erythrocytes. In each experiment a group of untreated birds and at least one group treated with a drug of known antimalarial activity served as controls.

The results of the experiments on trophozoite- and sporozoite-induced avian malaria are given in Table I. Whereas quinine, atabrine and sulfadiazine can be used interchangeably as suppressives for the trophozoite infections, only sulfadiazine has prophylactic activity against *P. gallinaceum*. It is apparent that boric acid at relatively high concentrations in the diet had a marked suppressive effect on both *P. cathemerium* and *P. gallinaceum* infections. Boric acid at high doses also showed definite prophylactic activity against *P. gallinaceum* infections.

Thus boric acid, unlike quinine and atabrine, possesses both suppressive and prophylactic activity. It should be noted, however, that the amounts of boric acid required to produce an effect on avian malaria closely approach toxic levels.

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Antifibrillating Action of N-Methyl-Dibenzyl-Amine and Some of Its Derivatives.

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Quinidine is the best known among the drugs effective against cardiac fibrillation induced by electrical stimulation or other agents. Other drugs have been reported, some with chemical structure resembling that of quinidine, and others entirely different, which also show some antifibrillating action.

We have found that α-fagarine, (an alkaloid isolated by Stuckert[1] from *Fagara coco* (collected by Gill Engler) exerts an antifibrillating effect as potent as, or more potent than, quinidine itself. It counteracts the influence of several fibrillating agents (faradic currents,[2,3] coronary occlusion[4]) and causes recovery of the sinus rhythm in cases of accidental or spontaneous auricular fibrillation[5] in animal experimentation or in man.[6,7]

Deulofeu and Labriola[6] have established that α-fagarine is a tertiary base to which the following tentative formula can be assigned:

[1] Stuckert, G., *Investigaciones de laboratorio de Química Biológica*, Córdoba, Vol. I, 1933; Vol. II, 1938.

[2] Moisset de Espanés, E., *Rev. Soc. Arg. Biol.*, 1937, **13**, 112; *C. R. Soc. Biol.*, 1938, **127**, 118.

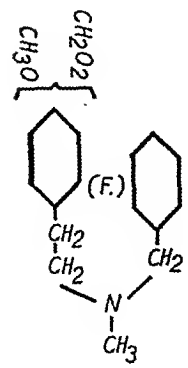
[3] Martinez, C., *Medicina* (Buenos Aires), 1944, **4**, 109.

[4] Moisset de Espanés, E., *Rev. Soc. Arg. Biol.*, 1937, **13**, 116; *C. R. Soc. Biol.*, 1938, **127**, 233.

[5] Moisset de Espanés, E., *Amér. Clín.*, 1945, **8**, 41; Moisset de Espanés, E., y Moyano Navarro, B., *Rev. Soc. Arg. Biol.*, 1936, **12**, 137; *C. R. Soc. Biol.*, 1938, **127**, 510.

[6] Deulofeu, V., Labriola, R., Orias, O., Moisset de Espanés, E., y Taquini, A., *Science*, 1945, **102**, 69; *Ciencia e Investigación*, 1945, **1**, 527.

[7] Taquini, A., *Rev. Arg. Cardiol.*, 1945, **12**, 53.



The possibility of preparing easily a series of bases derived from methyl-dibenzyl-amine, which has a chemical structure greatly resembling that of the possible structure of α-fagarine, and the hope of finding new antifibrillating substances which, because of efficiency or facility of preparation, would offer advantages, induced us to assay them pharmacologically. Methyl-dibenzyl-amine, which also exerts some antifibrillating effect, as shown by Martinez,[3] was taken as standard to compare the activity of its derivatives.

So far we have studied the following bases, arranged according to the increasing complexity of the substituents attached to the aromatic nuclei: (1) N-methyl-dibenzyl-amine; (2) N-methyl-o-anisyl-p-anisyl-amine; (3) N-methyl-di(o-anisyl)-amine; (4) N-methyl-di-(3,4-dimethoxy-benzyl)-amine; (5) N-methyl-di-(2,3-dimethoxy-benzyl) - amine; (6) N-methyl-di-(p-anisyl)-amine; (7) N-methyl - benzyl - piperonyl - amine; (8) N-methyl-di-(piperonyl)-amine; (9) N-methyl-p-anisyl-piperonyl-amine. The following bases were also studied: (10) N-ethyl-dibenzyl-amine; (11) N-methyl-benzyl-anisidine; (12) N-methyl-benzyl-phenethyl-amine. We shall refer to the above mentioned bases by the number preceding each. (Fig. 1).

All the bases were prepared under the direction of Doctors V. Deulofeu and R. Labriola in the Laboratory of Organic Chemistry in the Facultad de Ciencias Exactas, Fisicas y Naturales de Buenos Aires. Some are already known and the others were obtained by classical methods. The description of the new bases will be given elsewhere.

Rabbits weighing from 1200 to 1800 g were anesthetized with 0.6 g of dial per kg of body weight, half of that dosage being injected intravenously and the other half intraperitoneally. The heart was exposed, with the pleural sacs left untouched, and the thresholds to obtain both auricular and ventricular fibrillation with faradic current were determined. Control experiments showed that such thresholds do not show appreciable variations within the 2 or 3 hours following the beginning of anesthesia.

Two, 7, 17, and 32 minutes after intravenous injection of the substance to be tested, the threshold was again determined, and the maximal variation was taken as indicative of the activity of the drug for comparative purposes.

Table I summarizes the results obtained. The activity of each drug is expressed according to the percentage of threshold increase found after injection. Each figure is the average of the values found in the different animals injected with the same drug. The relative value represents the ratio of the activity of the investigated substance to that of the same dose of the standard substance (methyl-dibenzyl-amine), to which was assigned an activity value of 100. The opponent action of both quinidine and α-fagarine against auricular and ventricular fibrillation was also determined for comparative purposes.

The results show that methyl-dibenzyl-amine exerts antifibrillatory action and that its potency is about the same on auricular and ventricular fibrillation (Table I). The addition of either methoxylic or dioxymethylenic groups to the aromatic nuclei of methyl-dibenzyl-amine, obviously modified its effect. In some cases it decreased (bases 4 and 5) and in some others it increased (bases 3 and 9) but the change did not affect to the same extent the auricular and ventricular effects. All the bases to which only methoxylic groups were added (bases 2, 3, 4, 5, and 6) showed increased activity against ventricular fibrillation, whereas those to which only dioxymethylenic groups were added, (bases 7 and 8), showed increased activity against auricular fibrillation as compared with that of the original nucleus. Bases either having no such groups (bases 1, 10, 11, and 12) or hav-

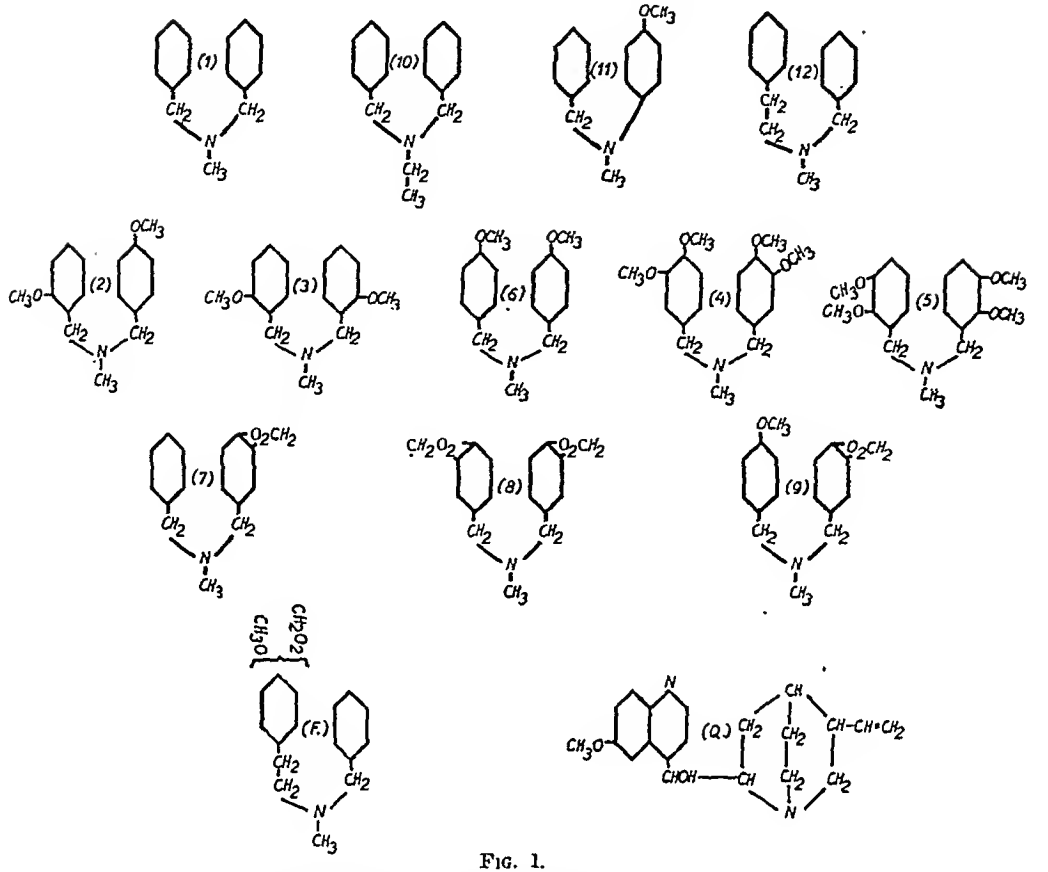


FIG. 1.

(1) N-methyl-dibenzyl-amine; (2) N-methyl-o-anisyl-p-anisyl-amine; (3) N-methyl-di(o-anisyl)-amine; (4) N-methyl-di-(3,4-dimethoxy-benzyl)-amine; (5) N-methyl-di-(2,3-dimethoxy-benzyl)-amine; (6) N-methyl-di-(p-anisyl)-amine; (7) N-methyl-benzyl-piperonyl-amine; (8) N-methyl-di-(piperonyl)-amine; (9) N-methyl-p-anisyl-piperonyl-amine; (10) N-ethyl-dibenzyl-amine; (11) N-methyl-benzyl-anisidine; (12) N-methyl-benzyl-phenetyl-amine.

ing both (one of each) (base 9) showed practically the same degree of activity against both auricular and ventricular fibrillation.

Ethyl-dibenzyl-amine (base 10) also was of interest because substances mentioned in the literature as having antifibrillatory activity have ethyl groups attached to nitrogen. In our substance the substitution of the ethyl for the methyl group rather reduced the activity of the standard substance.

Methyl-benzyl-phenetyl-amine (base 12) with a chemical structure even more closely resembling the proposed structure for α-fagarine than methyl-dibenzyl-amine, our standard substance, showed a somewhat higher activity than the latter.

Furthermore, outside the methyl-dibenzyl-amine series, the aromatic base methyl-benzyl-anisidine was investigated, and showed a markedly weak potency against both auricular and ventricular fibrillation, indicating that the difference in chemical structure produced a difference in pharmacological properties.

Among the substances with higher potency than our standard substance, base 2 (methyl-o-anisyl-p-anisyl-amine) showed a very transient effect (it vanished in approximately 5 minutes) and base 3 (methyl-di(oanisyl)-amine) was highly toxic, producing an intense convulsive attack, more tonic than clonic, the intensity in proportion to the administered dose, appearing sometimes before

TABLE I.

Table Showing the Increase of Faradic Threshold to Fibrillation in Per cent of Initial Value and the Potency of Its Activity as Compared with That of Methyl-dibenzyl-amine at the Same Doses.

		Auricles			Ventricles		
Drug	Dose, mg/kg	No. of animals	% of fibrillation threshold increase (avg and P.E.)	Relative activity	No. of animals	% of fibrillation threshold increase (avg and P.E.)	Relative activity
1	1	24	18.3 ± 1.11	100	20	18.4 ± 1.01	100
	5	29	27.1 ± 2.15	100	20	32.8 ± 2.37	100
2	1	10	18.5 ± 6.65	101	10	25.7 ± 6.74	140
3	½	8	25.0 ± 6.13	137	8	34.6 ± 4.12	188
4	1	11	13.3 ± 1.23	73	11	16.1 ± 2.99	87
5	1	12	10.0 ± 5.81	55	12	14.4 ± 4.32	78
6	1	12	10.2 ± 3.24	56	11	29.4 ± 2.21	160
7	5	10	31.0 ± 4.07	114	10	18.8 ± 6.87	57
8	5	9	29.2 ± 1.98	108	10	12.4 ± 3.81	38
9	1	12	20.3 ± 2.36	111	12	23.3 ± 1.48	127
10	5	10	22.7 ± 1.67	84	10	23.9 ± 3.78	73
11	5	12	1.8 ± 3.84	7	11	5.2 ± 1.36	16
12	1	11	22.3 ± 2.06	122	11	21.6 ± 2.02	117
Q	1	17	21.8 ± 2.87	119	12	16.8 ± 2.60	91
F	1	21	24.8 ± 2.55	135	12	30.3 ± 2.12	165

the injection ended and ceasing under new doses of "dial."

Even though quinidine showed some higher activity against auricular fibrillation, its potency was of the same order as that of our standard substance; α-fagarine, on the other hand, being perhaps more effective against ventricular than against auricular fibrillation, showed, in general, a higher potency than methyl-dibenzyl-amine.

It seems necessary to emphasize that a strict ratio does not exist between the increase in potency as compared with the increase of the administered dose, as generally occurs in pharmacology. Also the relation dose/effect is different for each substance of the series; therefore, the relative activity may not be the same if doses other than those we have used are compared. Furthermore the toxicity of one substance as compared with the others does not necessarily parallel antifibrillating activity. More study will be needed to indicate which one is the most convenient from the practical point of view.

Summary. The antifibrillating activity of a series of tertiary bases with a carbon skeleton related to the possible chemical structure of α-fagarine has been assayed in rabbits. Eight of them were derivatives of methyl-dibenzyl-amine by addition of either methoxylic or dioxomethylenic groups to the aromatic nuclei; the others, also chemically related, were ethyl-dibenzyl-amine, methyl-benzyl-anisidine and methyl-benzyl-phenetyl-amine. Their effects were compared with those of quinidine and α-fagarine, tested under the same conditions.

All of them showed antifibrillating activity. The maximal effect, however, was given by α-fagarine. The addition of methoxylic and dioximethylenic groups modified the activity of methyl-dibenzyl-amine, the former enhancing the effect against fibrillation of the ventricles and the latter enhancing the effect against auricular fibrillation.

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Plasma Levels after Repository Injections of Penicillin in Water-in-Oil Emulsions.

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A comparison of the penicillin levels obtained in the same patients after the same intramuscular dose of penicillin given in saline and in a water-in-oil emulsion as advocated by Freund and Thomson[1] was presented in a previous communication.[2] Some variations were noted in the maximum concentrations obtained and considerably greater differences were noted in the maintenance of levels in the blood after any given dose. Intramuscular doses of 100,000, 200,000 and 300,000 units were given. Higher and better sustained levels followed the larger doses. There was no constant or striking difference between the results obtained in the same subjects after the same dose given in the 2 vehicles. A dose of 300,000 units in a beeswax-peanut oil mixture gave maximum concentrations which were appreciably lower, but the levels were much better sustained than with the same dose given in saline or in the water-in-oil emulsion. The emulsion base used in the previous study was Pendil, a "chloresterol-derivative and peanut oil" as described by Freund and Thomson.

A second series of observations are reported here with the use of a similar preparation, "Emulgen,"* which is composed of "sesame oil and cholestrin base." Chlorobutanol 3% is added as a preservative and local anesthetic. The methods used were the same as in the previous studies. Each subject was given a single dose of 200,000 or 300,000 units in saline or in the water-in-oil emulsion and

[1] Freund, J., and Thomson, K. J., *Science*, 1945, 101, 468.

[2] Ory, E. M., Wilcox, C., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1946, 62, 86.

* Supplied by Dr. C. O. Miller, Lakeside Laboratories, Inc.

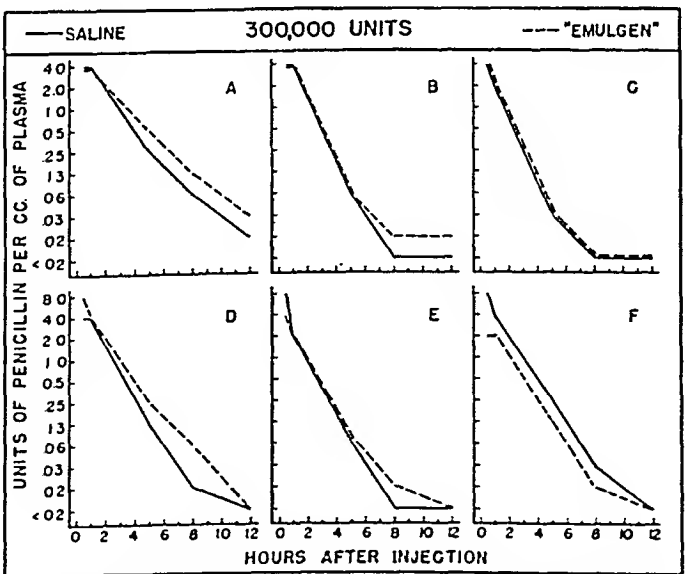

Fig. 1.

Plasma penicillin levels in 6 subjects after intramuscular injections of 300,000 units in saline and in a water-in-oil emulsion.

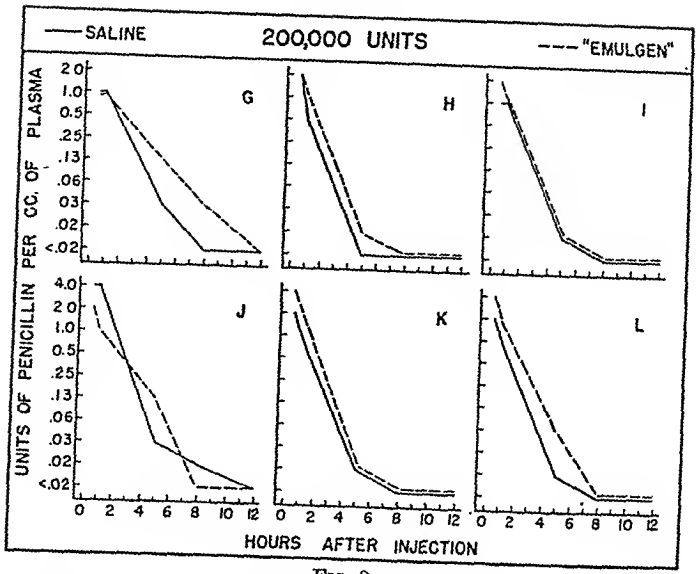

FIG. 2.
Plasma penicillin levels in 6 subjects after intramuscular injections of 200,000 units in saline and in a water-in-oil emulsion.

TABLE I.
Average Concentrations of Penicillin in Plasma Following Intramuscular Injections in Two Vehicles.

Hours after injection	Units per cc of plasma			
	After 200,000 units in		After 300,000 units in	
	Saline	"Emulgen"	Saline	"Emulgen"
½	2.00	2.50	5.33	4.33
1	1.42	1.33	3.33	3.00
5	0.016	0.06	0.13	0.17
8	0.003	0.005	0.02	0.04
12	0	0	0.003	0.008

the same dose was given to the same subjects 2 or 3 days later in the same final volume of the alternate vehicle. A single batch of penicillin was used in this study. This was a purified product which consisted almost entirely of crystalline penicillin G.† Special care was taken to obtain a smooth, creamy emulsion as advocated by Freund and Thomson. There was no local irritation from any of the injections. Blood for penicillin levels was drawn at ½, 1, 5, 8 and 12 hours after the injections. The results are shown graphically for each subject in Fig. 1 and 2 and the average levels for each dose level are given in Table I. Comparable, but not the same subjects were used for the 2 dosages.

As in the previous report, the maximum concentrations varied somewhat in different subjects, but there were greater variations in the rate of decline in the plasma levels. After a dose of 300,000 units, demonstrable levels were no longer present at 12 hours except in one subject. After 200,000 units, there was no demonstrable penicillin in the plasma after 8 hours in almost every instance. In about one-half of the subjects receiving each dose, the penicillin levels were slightly better sustained when given in water-in-oil emulsion. The average blood levels after 5 and 8 hours were also somewhat higher in subjects receiving the emulsion. Much more striking, however, is the fact that the plasma levels obtained after 300,000 units in saline

† Supplied by the Commercial Solvents Corporation.

were considerably higher and much better sustained than those observed after 200,000 units given in the same volume of the water-in-oil emulsion.

Conclusion. The previous observation that only a slight and inconstant prolongation of penicillin blood levels is obtained by the use of a water-in-oil emulsion is confirmed. The present findings suggest further that a substantial increase in the intramuscular dose of penicillin in the same volume of saline will give considerably higher and better sustained plasma levels than the smaller dose given in a water-in-oil emulsion and the disadvantages of the latter method are thus avoided.

15547

A Constant Current Square Wave Stimulator.

HENRY W. NEWMAN.

From the Department of Medicine, Stanford University School of Medicine, San Francisco, Cal.

In the study of neuromuscular excitability, both experimental and clinical, a source of stimulating electric current is required. As the tests employed have been refined, there has been simultaneous refinement and complication of the apparatus required to perform them, with the development of a multiplicity of stimulators, each designed for one specific test. It is the purpose of this paper to describe the construction of a stimulator which may be used both for the older and the more recent tests, yet which is not unduly complex, and which may be constructed from standard radio components.

This stimulator is capable of producing waves of essentially square form as seen in Fig. 1, with constant current at full output of 20 milliamperes so long as the output re-

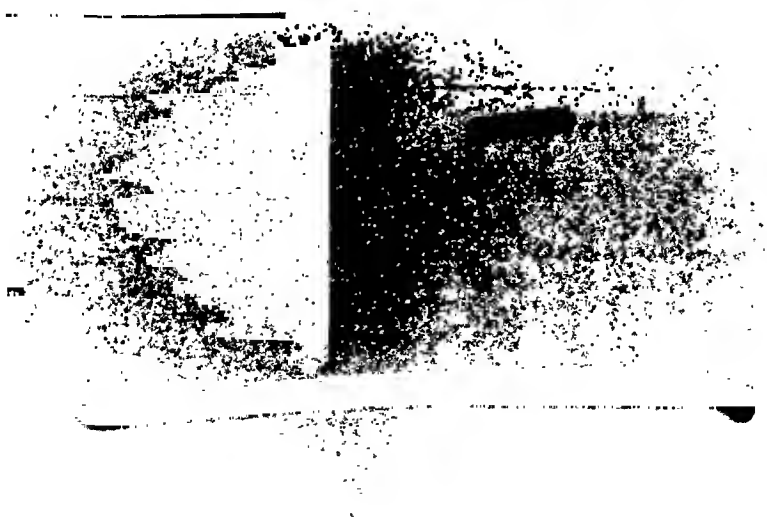

Fig. 1.

Cathode ray oscillographic tracing of stimulus of 5 milliseconds duration, showing square wave form.

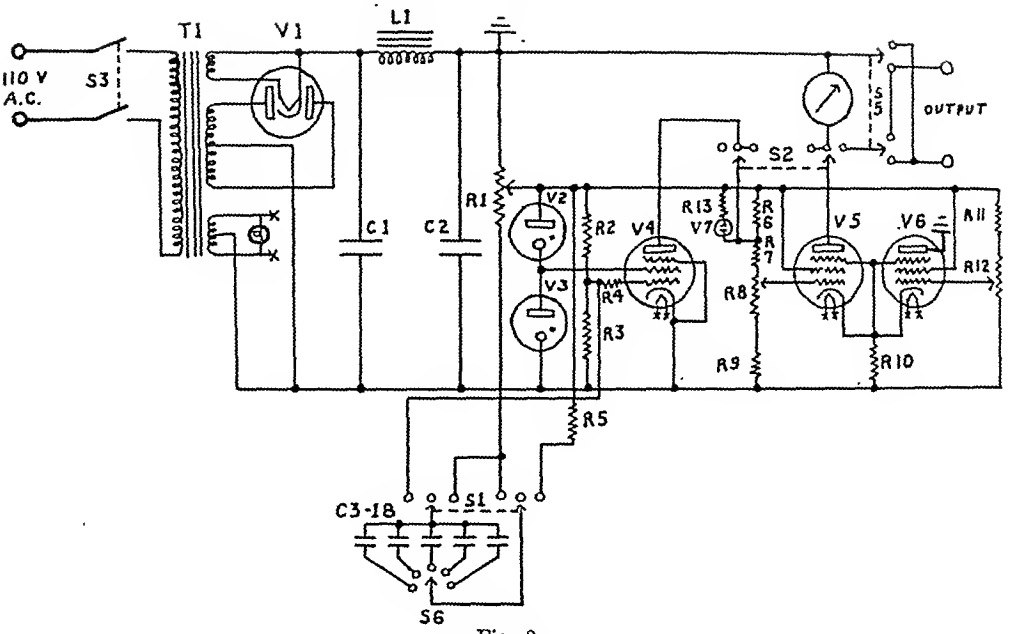

Fig. 2.

Schematic diagram of stimulator. The following components are required:

T1—Transformer, 800, 5.0, and 6.3 volts.
L1—Choke, 12 henrys at 100 ma.
C1, 2—8 fd. 600 valts.
C3 to 18—0.75, 0.5, 0.25, 0.1, .05, .025, .01, .005, .0025, .001, .0005, .00025, .0001, .00005, .000025, .00001 fd.
R1—20,000 ohms, 25 watts, voltage divider.
R2—6 megohms, ½ watt.
R3, 4—1 megohm, ½ watt.
R5—100,000 ohms, 1 watt.
R6—100,000 ohms, 5 watts.
R7—750,000 ohms, 1 watt.
R8—250,000 ohms, variable.
R9—80,000 ohms, 1 watt.
R10—1,500 ohms, 5 watts.
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V4—Pentode, 6SJ7.
V5, 6—Pentodes, 6F6.
V7—Miniature neon tube.
S1, 2—Ganged 2P3T switches.
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S6—1P16T selector switch.

sistance is kept below 15000 ohms. The duration of the stimulus may be varied in 16 steps from 0.02 to 1500 milliseconds, and its intensity accurately determined prior to administration by means of a conventional milliammeter. A switch provides for reversal of polarity. Thus it is possible to determine strength-duration curves, rheobase and chronaxia, relation between cathodal- and anodal-closing thresholds, and galvanic tetanus ratio.

The circuit, shown schematically in Fig. 2, is derived, with considerable modification, from a basic circuit described by Bauwens.[1] Automatic administration of shocks at fixed intervals was abandoned in favor of manual administration of single shocks, since this was found to be much better tolerated by human subjects. Grounding of the positive output terminal affords some protection from accidental shock.

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volts. This condition is satisfied so long as, with full output of 20 milliamperes, the output resistance does not exceed 15000 ohms, a condition easily met in practice.

By employing a common cathode resistance for tubes V5 and V6 their total plate current, and thus the load on the power supply, is kept constant whether a stimulus is being applied or not, eliminating the necessity for elaborate regulation of the power supply. Resistance R12 is adjusted so that with the intensity control R8 at maximum and switches S1 and S2, which are ganged, in the charging position, the milliammeter indicates 20 milliamperes. Thereafter R12 need not be adjusted further.

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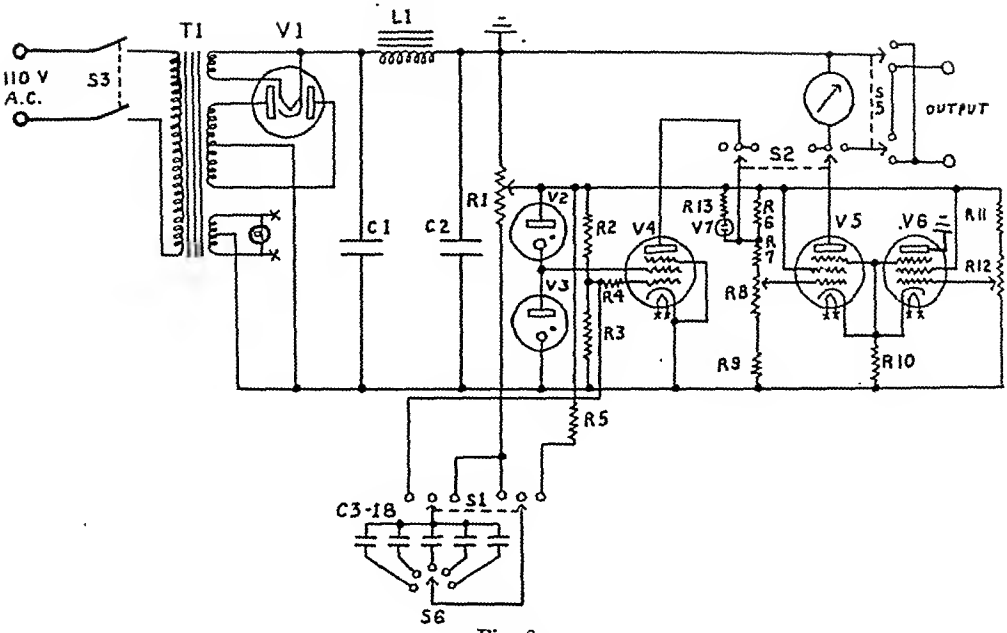
In practice, a large indifferent electrode is used, with the stigmatic electrode of 1.0 sq cm area placed over the nerve or motor point of the muscle to be tested. The threshold stimulus is then determined, first for currents of the longest duration available, and then for those of successively shorter duration until no further response can be obtained at maximum intensity. The values of threshold intensity so obtained are plotted against a logarithmic scale of duration, the result being a strength-duration curve. From this curve chronaxia may easily be determined. Galvanic tetanus ratio is arrived at by first establishing the threshold at which a simple muscle twitch is produced with a current of 1.5 seconds duration, and then increasing the intensity until a tetanic contraction persisting for the duration of the stimulus is secured. The ratio between these 2 intensities is the galvanic tetanus ratio.

Polarity of output is controlled by S3; the positive side remains at ground potential. Thus the relationship of anodal-closing to cathodal-closing thresholds may be easily obtained. In determination of galvanic tetanus ratio, observation of the small neon light, V7, which is extinguished during the passage of the stimulus, is helpful in determining the tetanus threshold, for which purpose it is mounted on flexible leads so that it may be observed at the same time as the contracting muscle.

Summary. A stimulator suitable for the determination of strength-duration curves, rheobase and chronaxia, ratio of cathodal to anodal thresholds, and galvanic tetanus ratio has been constructed of standard radio components. It has given reliable and reproducible results in cases of peripheral nerve injury.



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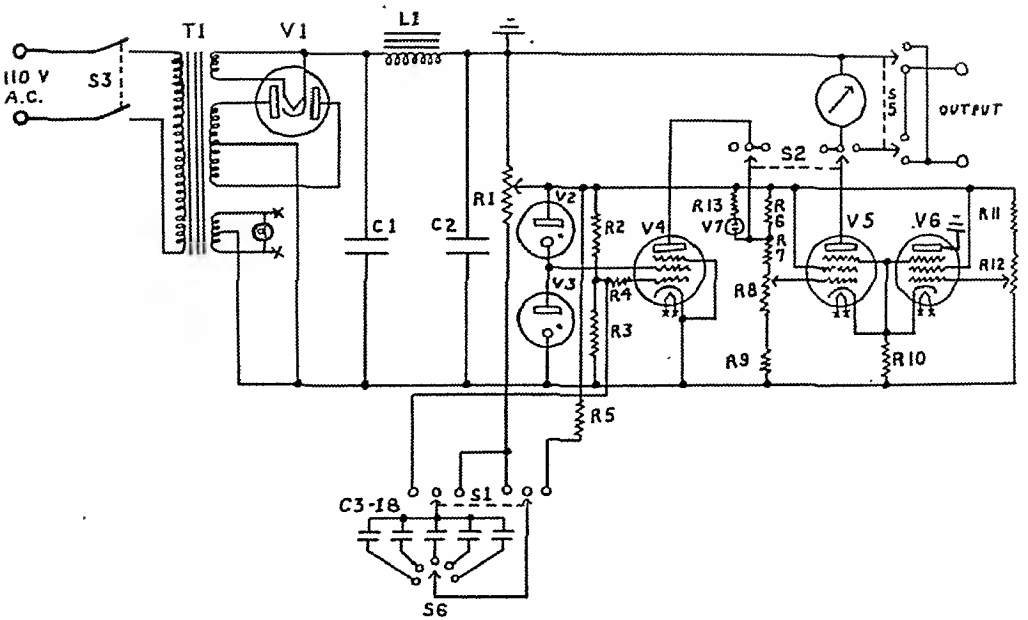

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15548

Effects of 2,4 - Dichlorophenoxyacetic Acid on Experimental Animals.*

NANCY L. R. BUCHER. (Introduced by Joseph C. Aub.)

From the Medical Laboratories, Collis P. Huntington Memorial Hospital, Harvard University, at the Massachusetts General Hospital, Boston, Mass.

2,4-Dichlorophenoxyacetic acid (2,4-D) is of biological interest because of its remarkable effects on plants. Its action is selective, not only with regard to different species of plants, but also with regard to the type of response in different plants and plant parts. It is a powerful growth stimulator in minute concentrations; actively growing plants are especially sensitive. Larger amounts produce morphologic changes which may occur at some distance from the point of application, and after an appreciable latent period, death of the plant ensues.[1-5]

In view of these properties it seemed worthwhile to investigate the effects of 2,4-D upon animals, with respect to toxicity, pharmacologic activity, and influence on normal and neoplastic growth. Plant hormones have previously been shown to inhibit the growth of transplantable tumors if applied directly to the tumor tissue,[6,7] but treatment of tumor-bearing animals by injection at a site remote from the tumor has been unsuccessful[7] except in rare instances.[8]

The sodium salt of 2,4-D was used in 1% solution in physiological saline, adjusted to approximate neutrality. Toxicity studies were carried out on young strain A male mice. The drug was injected either subcutaneously, intraperitoneally or intravenously and was found to be effective in the same general dose range by any of these routes. The drug has previously been reported to be nontoxic to animals and humans when administered orally.[4,5] In the present experiments the approximate LD_{50}, as determined by subcutaneous administration, was 280 mg per kg.

The injection of from 150 to 200 mg per kg at one time results in a myotonia-like syndrome that lasts from 8 to 24 hours or more in different animal species. The same signs have been produced in mice, rats, rabbits and dogs. Within a half to three-quarters of an hour following administration of the drug, the animal has ceased most spontaneous activity, and sits very still. However, he remains awake and alert. When he is now induced to make a sudden movement, such as a quick start, or a righting motion, spasm supervenes; his limbs spread out, and he falls and lies helpless, vainly trying to regain his footing. Opisthotonus may appear momentarily. The hind limbs are usually more noticeably affected than the fore limbs. If incited to continue his attempts to move, he will gradually recover. Motion is slow and awkward at first, but with continued effort approaches normal speed, smoothness, and control. After this, if the animal is allowed to remain still for 5 or 10 minutes, the initial syndrome recurs. In addition to the phenomenon of alleviation by exercise and exacerbation by rest, some animals exhibit a local hard knotty muscle spasm in response to a sharp percussive blow. Both features are characteristic of clinical myotonia. The diagnosis of myotonia has finally been established by myographic and electromyographic studies, now being done in conjunction with Dr. George Acheson of the Department of Pharmacology of the Harvard Medical School.

When larger doses (250 to 350 mg per kg)

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* This is publication No. 622 of the Cancer Commission of Harvard University.

This work was aided by a grant from the American Cancer Society, Massachusetts Division, Inc.

1 Zimmerman, P. W., and Hitchcock, A. E., *Contr. Boyce Thompson Inst.*, 1942, **12**, 321.

2 Hamner, C. L., and Tukey, H. B., *Bot. Gaz.*, 1944, **106**, 232.

3 Marth, P. C., and Mitchell, J. W., *Bot. Gaz.*, 1944, **106**, 224.

4 Hildebrand, E. M., *Science*, 1946, **103**, 465.

5 Mitchell, J. W., and Marth, P. C., *Bot. Gaz.*, 1944, **106**, 199.

6 Kline, B. E., Wasley, W. L., and Rusch, H. P., *Cancer Research*, 1942, **2**, 645.

7 Kline, B. E., and Rusch, H. P., *Cancer Research*, 1943, **3**, 702.

8 Tanaka, A., and Tuboi, S., *Gann*, 1940, **34**, 354.

are given to mice there is an initial period of myotonia as previously described, followed by rapidly increasing sluggishness and reluctance to move; tails are rigid; attempts at righting bring out a coarse clonic tremor. Inertia deepens into coma. The mice now lie on their backs with the 4 feet in the air. Their respirations are almost imperceptible. They are flaccid, and cold to touch. This state may terminate in death after several hours, or several days. In a few instances, after 8 to 12 hours, the mouse may go through the same stages in reverse order, finally recovering completely and without residua. Dosages of this magnitude have not been administered to other animals.

Other toxic effects of the drug which have been noted following parenteral administration are: (1) an irritant action on the eyes and nasal passages of dogs, resulting in sneezing spells, and violent rubbing of the eyes, which develops after a delay of 24 hours or more (2) gastro-intestinal disturbances in dogs manifested by vomiting and increasingly severe anorexia (3) the production of diarrhea in many, but not all mice.

Thus far no clear-cut chronic syndrome has been produced by continued administration of one or 2 daily injections to mice (1/3 to 1/5 of the LD_{50} per day for 3 weeks to 3 months).

Postmortem studies have not revealed any striking features peculiar to the drug. In acutely intoxicated mice the liver, kidneys and spleen grossly are dark and mottled. The upper part of the intestine is filled with a bile-colored liquid which is sometimes blood-stained. The bladder is full, and a mass of dry, hardened feces is caked about the anus. Microscopic examination shows wide dilatation of the blood vessels of lungs, liver and kidneys. In mice receiving prolonged treatment the only abnormalities noted have been attributable to infection. There was moderate atrophy of the liver in one chronically-treated dog, whose serum alkaline phosphatase rose from 5.0 to 39.4 Bodansky units in 3 weeks. This dog entirely refused food during the last week of life.

The peripheral blood of chronically-treated mice has not varied significantly from the controls with respect to hemoglobin, red cell, white cell and differential counts.

Small doses have not affected the growth rate of young mice. Larger amounts (1/4 of the LD_{50} daily) have retarded growth, probably through reduction of food intake. Mice who have survived this amount for 3 months are still alert, active and apparently normal in other respects. Repeated injections have not influenced the growth rates of 2 transplantable mouse sarcomas (Sarcoma 180 and Sarcoma 37) to any notable degree. Mitotic counts performed on these tumors at varying intervals after a single injection of 200 mg per kg did not differ significantly from the control values. Mice undergoing daily injections of 1/3 of the LD_{50} have become pregnant, and borne apparently normal litters at the end of a normal gestation period.

Summary. Parenterally-administered 2,4-D produces temporary myotonia in mice, rats, rabbits and dogs following a single injection. Repeated injections have failed to elicit either a characteristic chronic syndrome or a striking histologic picture; nor have they altered the process of reproduction and development in the dose ranges tested. The slight retardation of the growth rate of young mice on large daily doses is probably a manifestation of reduced food intake. Neoplastic growth has not been inhibited.

The author is grateful to Dr. Joseph C. Aub for the suggestion of this problem and for encouragement and advice. The technical help of Amory Glenn is also greatly appreciated.

15549

Effect of Some Atropine-like Drugs on Swing Sickness.

PAUL K. SMITH* AND ALLAN HEMINGWAY.†

From the Departments of Pharmacology and Biochemistry and of Biophysics, AAF School of Aviation Medicine, Randolph Field, Texas.

This study was undertaken primarily to find remedies of value in the prevention of airsickness. Any drug to be of value in the treatment of airsickness should fulfill the following criteria; it should be effective immediately following oral administration; it should not impair the capacity of treated personnel to perform duties; it should not be toxic, habit forming, or cause disagreeable symptoms.

Methods. The drugs tested included atropine, scopolamine, hyoscyamine, homatropine, benzoyltropine, benzoyloscine, and pavatrine (B-diethylaminoethyl fluorene-9-carboxylate hydrochloride). All drugs or lactose placebos were contained in No. 1 pink capsules. The drug was given approximately one hour before swinging.

The swing test consisted of being swung in the sitting position through an arc of 150° on a swing with a radius of 14 feet from the center of the swing to the seat. The head was fixed with the plane passing through the lateral canthus of each eye and the external auditory meatus of each ear horizontal to the ground when the swing was at rest. Swinging was continued until the subject vomited or for a maximum of 20 minutes. Further details of the procedure have been described[1] in which it was shown also that there was a fair correlation between swing sickness and airsickness.

Drugs were studied in 3 ways: In the first, unselected subjects were swung and those that vomited within 20 minutes were used later for tests of the drug or placebo. In the second procedure, the subjects who vomited the first time were divided into 2 groups and before they were swung the second time half were given the drug and half placebos while the third time the order was reversed. In the third procedure, the subjects before having been swung were given either the drug or placebo and the incidence of vomiting in the various groups recorded.

The subjects were all either aviation cadets or aviation students. When the same subject was used repeatedly an interval of several days was allowed to elapse between swings and attempts were made to equalize the average interval between swings for the groups receiving drugs and groups receiving placebos. This was done because subjects swinging repeatedly with short intervals between may become accustomed to the motion.

The tests for side effects were chosen because they might reveal undesirable effects, such as dry mouth, interference with normal vision, etc. The subjects used for studies of these effects were selected without regard to susceptibility to swing sickness. Before being given a drug, each subject lay quietly for at least 5 minutes at which time his pulse rate and blood pressure were measured. Each man was given some paraffin to chew and told to salivate as much as possible into a small graduated cylinder during a 4-minute interval. The near point of accommodation was measured by means of a Prince rule. In this procedure, a piece of paper with a fine black line on it was first moved out from the eye until the line was no longer blurred and then from out to near the eye until the line appeared blurred. The mean of these 2 measurements was recorded. Each eye was tested separately. The drug or placebo was then given and 1½ hours later the observations were repeated.

Results. The results of the swing tests are given in Table I. They have been analyzed merely on the basis of the percentage of subjects in each group that vomited. Of the drugs tested scopolamine hydrobromide, 0.75

* Now at Department of Pharmacology, George Washington University School of Medicine.

† Now at Department of Physiol., University of Minnesota School of Medicine.

1 Hemingway, A., submitted for publication.

TABLE I.
Swing Tests on Subjects After Atropine-like Drugs.

		Drug		Placebo		Estimated protection	
	mg	No. Tested	No. Vomited	No. Tested	No. Vomited	%	P§
Scopolamine hydrobromide	0.5	21‡	5	21	9	44	0.18
" "	0.75	21*	5	21*	10	50	0.11
" "	0.75	21‡	9	21†	20	55	<0.01
Atropine sulfate	1.0	20‡	11	21†	20	42	<0.01
l-Hyoscyamine hydrobromide	1.0	20‡	7	21†	20	63	<0.01
Homatropine hydrobromide	12.0	20‡	14	21†	20	26	0.03
Benzoyltropine hydrochloride	50.0	20‡	14	21†	20	26	0.03
Benzoyloscine hydrochloride	50.0	20‡	13	21†	20	32	0.02
Demerol	100.0	8‡	7	21†	20	10	0.46
Pavatrine	250.0	20‡	11	21†	20	42	<0.01
"	250.0	52	18	365†	98	—	>1.00
Scopolamine hydrobromide	0.75	146	16	365†	98	59	<0.01
Atropine sulfate	1.0	106	15	365†	98	47	0.01

* Subjects acted as own controls, receiving the drug the second or third time swung and the placebo the other time.

† These subjects receiving placebos were used at the same time several of the drugs were being studied and are grouped together.

‡ These subjects receiving drugs and the corresponding control group vomited on the initial selection swing.

§ Probability that observed difference could have occurred by chance.

TABLE II.
Parasympathetic Depressant Effects of Drugs.
Differences and standard deviations of differences between effects 1½ hours after administration of the drug and prior to administration.

Drug	No. subj.	Salivation		Pulse rate		Systolic blood pressure		Diastolic blood pressure		Accommodation	
		cc	S.D.	per min	S.D.	mm Hg	S.D.	mm Hg	S.D.	cm	S.D.
Placebos	58	1.0	1.8	— 9.6	6.0	—5.9	4.6	—2.8	5.8	—0.1	0.8
Scopolamine	20	—0.8	1.5	—15.4	4.0	—9.7	8.5	—3.0	6.5	0.4	1.4
l-hyoscyamine	20	—4.4	2.4	— 0.8	11.6	—5.2	5.6	—1.2	6.4	0.6	1.4
Pavatrine	23	0.5	2.6	— 9.9	4.7	—4.4	6.7	—0.9	4.6	—0.1	0.8
Atropine	20	—2.1	2.3	— 9.6	5.8	—4.8	8.2	—1.5	6.4	0.2	0.6
Benzoyltropine	21	—0.3	2.7	—10.8	4.0	—5.2	5.3	—0.1	4.6	0.3	1.0
Benzoyloscine	20	0.5	1.4	— 9.2	6.3	—5.7	5.2	—0.4	4.9	—0.1	0.9
Homatropine	20	—0.3	2.4	—13.2	4.4	—6.4	7.4	—0.3	7.0	0.6	1.6

mg, and atropine sulfate, 1.0 mg, were effective by 2 methods. Hyoscyamine hydrobromide, 1.0 mg, was effective by the only method it was studied. (The probability of the difference occurring by chance was less than 1 in 100). Homatropine, benzoyltropine and benzoyloscine were also moderately effective (the probability of the difference occurring by chance was less than 1 in 20). The effectiveness of Demerol was negligible and in some subjects caused nausea before they were swung. The study on Pavatrine in unselected subjects failed to confirm the earlier work on selected subjects and the incidence of vomiting after Pavatrine was even slightly higher than after lactose. There were no obvious factors to account for this difference. From the calculation of the estimated protection, it is obvious that not all of the drugs were of equal value but due to the limited number of subjects it was not possible to determine whether or not statistically valid difference in effectiveness existed. In general it may be said that scopolamine, atropine, and *l*-hyoscyamine were effective whereas homatropine, benzoyltropine and benzoyloscine were of a lower order of usefulness.

With the exception of Pavatrine, comparable results were obtained by the methods

when the subjects were selected and when they were unselected. This is reflected in the estimated protection of 50% and 55% with scopolamine in selected subjects and 59% in unselected subjects and in the 42% estimated protection with atropine in selected subjects compared with 47% in unselected subjects.

The effects of these drugs and some others on salivation, pulse rate, blood pressure and accommodation are presented in Table II. As was to be expected the resting subjects showed a slight decrease in blood pressure whether receiving the drug or placebo but there were no significant differences. Because of the biphasic action of the atropine-like drugs in first stimulating and then decreasing the effects, it was not surprising that there was considerable variation in the pulse rate. The effects were more pronounced for hyoscyamine where in some measurements the pulse rate taken 45 minutes after the drug was given revealed a mean decrease but after 1½ hours the mean rate was significantly higher than in the placebo group (probability of no difference less than 0.01). In the doses used, scopolamine and homatropine produced significantly greater decreases in pulse rate than the placebo. In all cases the probability of no difference was less than 0.01. Significant decreases in salivary flow (probability of no difference less than 0.01) were produced by hyoscyamine, atropine and scopolamine. The effect produced by hyoscyamine was greater than that produced by any other drug tested and produced a definitely dryer mouth than scopolamine alone (probability of no difference less than 0.01).

Discussion. Not enough compounds have been tested to show clearly the relation between chemical structure and effectiveness in motion sickness. From the limited data it appears that substitution of the tropic acid group, as it appears in atropine, hyoscyamine and scopolamine with a mandelic acid group, as it occurs in homatropine or a benzoic acid group as it occurs in benzoyltropine and benzoyloscine, results in a great loss of activity.

In general, there is a rough correlation between the inhibition of salivation and the effectiveness in motion sickness. That is, scopolamine, hyoscyamine and atropine all depress salivation appreciably in the doses employed; whereas, the other drugs tested do not produce appreciable decreases nor are they significantly effective in motion sickness. The effects of these drugs on respiration were not studied but it would appear from earlier work that in general, atropine, scopolamine and hyoscyamine are more effective in stimulating respiration than the other drugs that were studied. Although this correlation between salivary depression and effectiveness in swing sickness may be of some value it should be pointed out that the effect on salivation of scopolamine is significantly less than that of hyoscyamine whereas there was no suggestion that hyoscyamine was more than slightly superior to scopolamine in swing sickness in the doses employed.

Summary. Scopolamine, atropine, hyoscyamine are effective remedies in preventing motion sickness due to swinging whereas homatropine, benzoyltropine and benzoyloscine were less effective and demerol and pavatrine were of doubtful value. None of the remedies, in the doses employed, produced effects on the pulse rate, blood pressure or near point of accommodation. Significant decreases in salivary flow were produced by atropine, hyoscyamine and scopolamine.

15550

Effect of Various Substances on Swing Sickness.

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The substances selected for study were chosen for the following reasons: The sodium barbital, sodium ethyl-allyl-thiobarbiturate (V-5),[2] Army Motion Sickness Preventive and thiamine had been suggested as possible remedies for motion sickness; the combinations of scopolamine and ethyl-B-methyl-allyl-thiobarbituric acid (V-12)[2] and of scopolamine, chlorobutanol and benzedrine had been suggested as mixtures of greater effectiveness than scopolamine alone; the mixture of scopolamine and neostigmine might retain the effectiveness in swing sickness of scopolamine[1] without the undesirable side effects;[1] the pyridoxine because it has been reported to be of value in the vomiting of pregnancy[3] and of irradiation sickness;[4] the benzedrine because it has sometimes been employed with the barbiturates to overcome the central depressant effects; and the sulfadiazine because it had been suggested as a mass prophylactic for the prevention of respiratory infections and information was desired as to whether or not it increased susceptibility to motion sickness.

The Army Motion Sickness Preventive consists of 65 mg sodium amytal, 0.40 mg atropine sulfate and 0.16 mg scopolamine hydrobromide per capsule. In this study the equivalent of 2 capsules of the Army Motion Sickness Preventive was used as a single dose.

Sulfadiazine was given to a group of subjects every day for 7 weeks. The subjects were tested at the end of the 4th and at the end of the 7th week. A similar group received placebos for a similar period of time. All remedies except the sulfadiazine were given in No. 1 pink gelatin capsules, approximately one to 2 hours before swinging began. The method of testing susceptibility of subjects to swing sickness is the same as in the previous report.[1]

The results are shown in Table I. The sodium barbital, benzedrine, V-5, thiamine and pyridoxine were of negligible effectiveness. The results with pyridoxine are not inconsistent with its possible value in the nausea of pregnancy and of irradiation sickness since the latter may be associated with liver damage. The Army Motion Sickness Preventive is moderately effective as might be predicted from its content of atropine and of scopolamine.[1] The number of subjects employed with sulfadiazine is so small that the suggested effectiveness is of no statistical significance. The data do suggest, however, that sulfadiazine in this dose does not appreciably increase the susceptibility to motion sickness. Inconsistent results were obtained with the mixture of scopolamine and neostigmine. In the group of selected subjects the mixture was considerably more effective than in the group of unselected subjects. The explanation for this is not apparent. Perhaps of more importance, however, is the observation that the mixture seems to retain some of the motion sickness preventive value of the scopolamine alone. A study of the effects of this mixture on salivation conducted as in the previous report showed that the neostigmine had abolished almost entirely the depressant effect on salivation. The mixture of scopolamine and V-12 is no more effective than the scopolamine alone was found to be.[1] The addition of chlorobutanol and benzedrine to scopolamine gave a remedy that was highly effective but the observed superiority of this mixture over scopolamine alone was not statistically signi-

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[1] Smith, P. K., and Hemingway, A., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 206.

[2] Noble, R. L., *Proc. Assn. Comm. Army Med. Res., N.R.C. Canada*, 1943.

[3] Willis, R. S., Winn, W. W., Morris, A. T., Newson, A. A., and Massey, W. E., *Am. J. Obs. Gyn.*, 1942, 44, 265.

[4] Maxfield, J. R., Jr., McIlwain, A. J., and Robertson, J. E., *Radiology*, 1943, 41, 383.

when the subjects were selected and when they were unselected. This is reflected in the estimated protection of 50% and 55% with scopolamine in selected subjects and 59% in unselected subjects and in the 42% estimated protection with atropine in selected subjects compared with 47% in unselected subjects.

The effects of these drugs and some others on salivation, pulse rate, blood pressure and accommodation are presented in Table II. As was to be expected the resting subjects showed a slight decrease in blood pressure whether receiving the drug or placebo but there were no significant differences. Because of the biphasic action of the atropine-like drugs in first stimulating and then decreasing the effects, it was not surprising that there was considerable variation in the pulse rate. The effects were more pronounced for hyoscyamine where in some measurements the pulse rate taken 45 minutes after the drug was given revealed a mean decrease but after 1½ hours the mean rate was significantly higher than in the placebo group (probability of no difference less than 0.01). In the doses used, scopolamine and homatropine produced significantly greater decreases in pulse rate than the placebo. In all cases the probability of no difference was less than 0.01. Significant decreases in salivary flow (probability of no difference less than 0.01) were produced by hyoscyamine, atropine and scopolamine. The effect produced by hyoscyamine was greater than that produced by any other drug tested and produced a definitely dryer mouth than scopolamine alone (probability of no difference less than 0.01).

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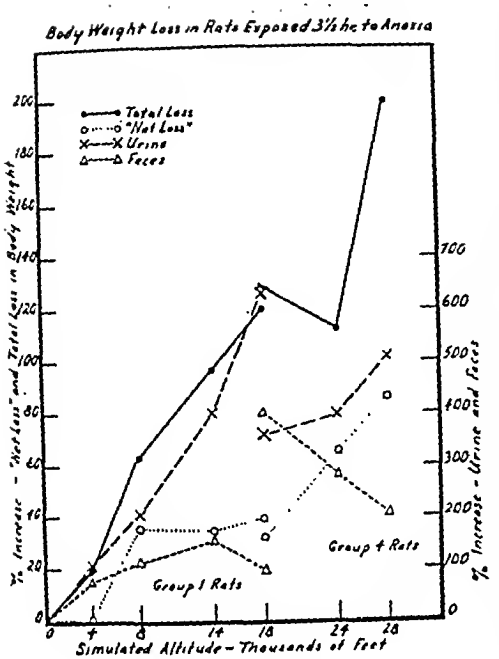
Summary. Scopolamine, atropine, hyoscyamine are effective remedies in preventing motion sickness due to swinging whereas homatropine, benzoyltropine and benzoyloscine were less effective and demerol and pavatrine were of doubtful value. None of the remedies, in the doses employed, produced effects on the pulse rate, blood pressure or near point of accommodation. Significant decreases in salivary flow were produced by atropine, hyoscyamine and scopolamine.



content of various organs is being studied by various workers, it seemed that information concerning total body weight losses occurring throughout the range of anoxia compatible with life would be helpful. An analysis of this weight loss into its components: urine, fecal and insensible water loss as Swann and Collings have made, is quite instructive, so this was done also. Furthermore, this procedure reveals the effect of anoxia on the secretion of urine, a problem which is not yet fully understood.

Proccdure. Separately weighed rats were placed in individual metabolism cages in a decompression chamber for periods of 3½ hours; temperature and humidity within the chamber were determined. At the end of this period, the rats were weighed and the weight of feces and the volume of urine were determined.

The 6 rats constituting Group 1 were 4 months old (average wt 291 g) and were maintained on Purina Dog Chow. They were exposed to simulated high altitudes not oftener than twice a week and during a 5-month period received exposure to altitudes of 4,000, 8,000, 14,000, and 18,000 ft. Between exposures to altitude, control experiments were carried out with all factors the same except that the pressure was not reduced. The 6 rats in Group 4 were 3 months old (average wt 248 g) and were kept on Rockland Rat Diet—Complete. During the course of 6 months and in similar experiments these rats were exposed to 18,000, 24,000 and 32,000 ft. Before the final 4 experiments at 28,000 ft. they each received 1 mg cocaine hydrochloride intraperitoneally.

Results and Discussion. In the figure, the results are expressed as per cent change from the control at each altitude. In addition to the changes in total weight loss, urine secretion and fecal excretion; changes in "net loss" are indicated. "Net loss" is the difference between the sum of the weights of feces and urine (specific gravity assumed: 1.0) and the total body weight loss. This value actually includes losses by evaporation from voided urine and feces as well as from the lungs and skin. But since the error due to urine and fecal evaporation is controlled when experimental values are compared with control values, the changes in "net loss" must represent fairly well those in insensible water loss. The same argument is applicable to changes in urine and feces.

The average temperature in all determinations varied between 24.3° and 25.1°C. The greatest difference in average relative humidity between control and experimental determinations (range of differences: 1.5-17.0) was at 24,000 ft. where total loss is out of line, but in the opposite direction expected. Apparently other factors than those we attempted to control were of more importance.

The average body weight loss in 16 control determinations on the rats of Group 1 was 13.9 mg/g body weight; of this 2.7 mg/g was urine; 2.3 mg/g was feces and 8.9 mg/g was "net loss." Similar results were found with the rats of Group 4.

The threshold altitude for each variable was found by determining which differences between the control and experimental values were statistically significant by the method of Fisher.[3] The thresholds for increases in

[3] Fisher, R. A., *Statistical Methods for Research Workers*, fourth edition, Oliver and Boyd, London, 1932.

TABLE I.
Swing Tests on Subjects After Various Substances.

		Drug		Placebo			
	mg	No. Tested	No. Vomited	No. Tested	No. Vomited	Estimated protection %	P§
Barbital, sodium	325	20	12	20	9	—	1.00
Benzedrine sulfate	10	20	13	20*	14	7	0.73
V-5	250	21	13	21*	15	13	0.52
Thiamine chloride	10	20	12	20*	14	14	0.66
USAMSP		19	11	19*	16	31	0.07
Sulfadiazine, 1 g†		19	4	18	4	0	1.00
Sulfadiazine, 1 g‡		19	5	17	7	36	0.33
Hyoscine hydrobromide prostigmine bromide	0.75 15.0	20	10	21	20	48	0.01
Hyoscine hydrobromide prostigmine bromide	0.75 15.0	81	17	365	98	22	0.28
Pyridoxine hydrochloride	100	33	15	82	30	—	1.00
Pyridoxine hydrochloride	200	28	9	82	30	12	0.66
Hyoscine hydrobromide V-12	0.75 250	104	14	365	98	50	0.01
Hyoscine hydrobromide chlorbutanol benzedrine sulfate	0.75 500 10	145	14	365	98	64	0.01

* Subjects acted as own controls, receiving the drug the second or third time swung and the placebo the other time.
† Tested after receiving the drug for 4 weeks.
‡ Tested after receiving the drug for 7 weeks.
§ Probability that observed difference could have occurred by chance.

ficant.

Summary. 1. Sodium barbital, benzedrine, V-5, thiamine, and pyridoxine were of no demonstrated value in the prevention of swing sickness. 2. Sulfadiazine in daily doses of 1 g did not appreciably affect the susceptibility to swing sickness. 3. A mixture of sodium amytal, atropine, and scopolamine was effective in swing sickness but probably not significantly more effective than could be predicted from its content of atropine and scopolamine. 4. The addition of neostigmine to scopolamine decreased the effectiveness of this mixture over that of scopolamine alone but did not entirely abolish it, although it effectively prevented the depressant action of scopolamine on salivation. 5. The addition of the thiobarbiturate V-12 to scopolamine did not increase its effectiveness in swing sickness. 6. A mixture of scopolamine, chlorobutanol and benzedrine was insignificantly more effective than the scopolamine alone in a similar dose.

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Effect of Anoxic Anoxia on Body Weight Loss in Rats.

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Fasting rats lose a significantly greater amount of weight when exposed to simulated altitudes of 8,000, 18,000 and 28,000 ft. than at normal barometric pressure.[1,2] Since the effect of anoxia on the weight and the water

1 Van Liere, E. J., *Anoxia, Its Effect on the Body*, The University of Chicago Press, Chicago, 1942.

2 Swann, H. G., and Collings, W. D., *J. Aviation Med.*, 1943, 14, 114.

TABLE I.

Animal No.	Wt, kg	Total amt DDT in mg/kg	Days injections given	Onset and duration of symptoms
263	2.1	370	1, 8, 11, 18, 28	Onset: 30th day Death: 33rd "
264	1.6	665	1, 8, 18, 28, 33, 39, 78, 85, 92	Onset: 232nd " Death: 235th "
287	1.5	200	1, 8	Onset: 13th " Death: 16th "
288	2.4	275	1, 31, 38, 45	Onset: 114th " Death 116th "
289	2.0	100	1	Died 3rd day without neurological signs
291	4.0	135	1, 13, 23	Onset: 42nd day Death: 45th "
292	2.2	225	1, 13, 23	Onset: 29th " Death: 29th "

on definite portions of the nervous system, such as other drugs possess.

Seven normal adult cats were given intramuscular injections of DDT solution in olive oil. This way was chosen with the belief that it afforded, as compared to the oral route, a better control of dosage and a prolonged absorption time. By slower absorption, we hoped to produce less liver damage, and to be able to observe more easily the effects on the nervous system. It was hoped to obtain *chronic animals*, but, even though the dosage was far below the lethal amount, the animals either quickly developed steadily increasing signs of intoxication, ending in death, or, after transient illness, recovered completely.

The data gathered from our experiment are summarized in Table I. The occurrence of transient symptoms is not represented.

When using the same amount for each individual injection, and the same interval between injections, we observed in our animals a great variability of response. One animal died after only 135 mg per kg, in 3 injections, while another remained in good condition after having received a total dose of 665 mg/kg.

Very often, if not always, when the first signs of neurological disorder appeared, the animals exhibited signs resembling those of a cold: congestion and hypersecretion of the eyes and nose, without any rise in temperature. We could not decide whether these were autonomic effects of intoxication, the manifestation of a latent infection brought to light by the decreased resistance of the animals, or whether the occurrence of the neurological disorders was promoted by an occasional infection. This seems unlikely, since in our animals these symptoms resembling a cold and the neurological disorders always occurred simultaneously.

A few days (between 3 and 6) after the last injection, the animals began to show definite stiffness, especially in the hind limbs. Very soon afterward a fine tremor developed, first seen only in certain postures and movements, but rapidly becoming more coarse and permanent. If the animals recovered, after a few days (usually less than a week), the symptoms progressively disappeared and never went any further than this tremor.

In other cases, after 2 or 3 days, the tremor increased in amplitude and became constant, while the stiffness extended to the proximal muscles of the limbs and to the trunk. At that period the animal continued to eat well and was still able to walk. The only sensory change noticed was obvious hyperesthesia. After that the clinical picture changed very rapidly. Muscular twitching appeared, first in the face, and soon became generalized. The animal could not walk, but retained consciousness. Within one day, the twitching became grosser, with resultant clonic movements of the entire limb. Finally the condition resembled status epilepticus, and the animal died. Injections of barbiturates may prevent the clonic movements for a short

total loss, "net loss" and feces were between 4,000 and 8,000 ft.; for urine below 4,000 ft.

The curves for per cent increase in total weight loss and "net loss" for the 2 groups of rats are fairly consistent with each other and together indicate increases proportionate to the altitude. The rats of Group 4 did not display as intense a polyuria in response to anoxia as those of Group 1, but they, nevertheless, had an increase in urine secretion which was proportionate to the altitude. The rats of Group 4 had a greater increase in fecal excretion at all altitudes than those of Group 1. These differences between the 2 groups were most probably due to the character of the diet; that for Group 1 being inferior. The eventual decline of the fecal excretion curves with altitude was probably due to a reduced intake of the diet, since loss of appetite is a well known symptom of anoxia.[1] The increased fecal excretion confirms the impression gained in this laboratory that in dogs, also, defecation is frequently one of the responses seen at 28,000 ft. It is of interest that colonic motility as registered by an enterograph in barbitalized dogs is usually greatly reduced by anoxia.[4]

Swann and Collings,[2] who subjected their rats under different conditions to 18,000 ft. for periods of time between 6 and 23 hours, found much greater increases in the rate of insensible water loss than reported here. This may explain in part their failure to find a diuresis at high altitude. Time may be a factor also, because their results for an exposure of 23 hours were less than those at shorter exposures and practically no different than the controls. Their finding an increase in the rate of fecal loss during the 6-hour exposure only, is to be expected in fasting rats.

The increases, here reported, in urine secretion are even greater than those found by Silvette.[5] The few experiments in which cocaine was administered did not reveal increased urine secretion above that seen without this epinephrine-potentiating agent, nor did they reveal a reduced secretion. This result was obtained in spite of the fact that cocaine caused in the control experiments, an increase of over 200% in urine secretion. Such results are not favorable to the view that anoxia-induced changes in urine secretion are a result alone of epinephrine and/or augmented sympathetic impulses to the kidneys. Such an explanation is more in accord with the results found in anesthetized animals[6] where oliguria is frequently found as well as polyuria in response to anoxia. Furthermore, 32,000 ft. proved lethal to all but one of the rats, but in spite of this severe stimulation of the autonomic nervous system, they continued to respond with polyuria.

Summary. The body weight loss in rats is proportional to the altitude up to 28,000 ft. The thresholds for increased total body weight loss, "net loss" (insensible water loss) and fecal excretion lie between 4,000 and 8,000 ft.; that for urine, below 4,000 ft. The administration of cocaine did not change the urinary response to anoxia (28,000 ft.)

[4] Van Liere, E. J., Northup, D. W., Stickney, J. C., and Emerson, G. A., *Am. J. Physiol.*, 1943, **140**, 119.

[5] Silvette, H., *Am. J. Physiol.*, 1943, **140**, 374.

[6] Toth, L. A., *Am. J. Physiol.*, 1940, **129**, 532.

15552

Neural Effects of DDT Poisoning in Cats.

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The literature of the last few years contains several reports[1,2] of nervous disorders produced in laboratory animals by DDT poisoning. Our aim was to investigate whether DDT has, to any extent, a selective action

[1] Lillie, R. D., and Smith, M. I., *Pub. Health Rep.*, 1944, **59**, 979.

[2] Nelson, A. A., Draize, J. H., Woodard, G., Fitzhugh, O. G., Smith, R. B., Jr., and Calvery, H. O., *Pub. Health Rep.*, 1944, **59**, 1009.

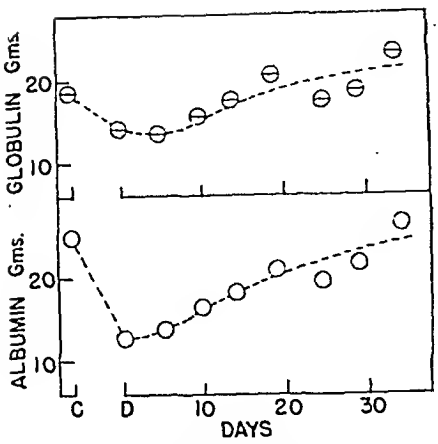

FIG. 1.

Total circulating plasma albumin and globulins in dog No. 68 before (C) and after depletion in proteins (D) and during 35 days of feeding 0.35 g of casein hydrolysate nitrogen/day/kilo of body weight.

depletion have been described previously.[5] During the repletion period 2 dogs were fed orally a casein hydrolysate, and 2 were fed a lactalbumin hydrolysate,† each dog receiving 0.35 g of nitrogen/day/kg of body weight. The plasma and "available fluid" volumes were determined according to the technics described by Gregersen and Stewart.[6] Nitrogen determinations on the blood and urine were made using the Pregl Micro-Kjeldahl. The albumin and globulin fractions were determined by the salt fractionation method of Howe.[7]

Urine ammonia and urea nitrogen were determined by the aeration method of Van Slyke and Cullen,[8] creatinine and creatine were determined by the alkaline picrate procedure of Folin,[9] and uric acid was analyzed by the indirect method of Folin.[10] Urine alantoin was determined by the procedure of Young and Conway[11] and the ninhydrin-carbon dioxide method of Van Slyke, MacFadyen and Hamilton[12] was used to determine the α-amino nitrogen.

Results. The data in Fig. 1 illustrate the

TABLE I.

Data Obtained on 4 Dogs Before and After Depletion in Proteins and After Repletion from Feeding 0.35 g of Nitrogen per Kilo of Body Weight for 30 Days. Dog 28 and 65 received a lactalbumin hydrolysate and Dogs 42 and 68 a casein hydrolysate during the 30-day repletion period.

Dog No.	Wt, kg	Plasma protein, g %	Plasma vol., (P) ml	Available fluid (A) ml	A/P
		Control.			
28	10.5	6.75	444	3100	7.0
65	10.8	7.14	475	3000	6.3
42	12.1	6.90	430	2600	6.0
68	12.0	7.04	625	3500	5.6
		Depleted.			
28	10.7	4.52	373	3500	9.3
65	11.1	4.50	410	4400	10.7
42	10.6	4.72	400	3600	9.0
68	11.1	5.20	502	4400	8.7
		Repleted.			
28	11.4	6.50	443	2800	6.3
65	11.7	6.51	626	3200	5.1
42	10.3	6.80	465	2600	5.6
68	11.4	7.07	600	3400	5.6

[5] Seeley, R. D., *Am. J. Physiol.*, 1945, **144**, 369.

† These hydrolysates were prepared by Dr. Bacon F. Chow of the Squibb Institute for Medical Research. Details concerning these hydrolysates will be published elsewhere.

[6] Gregersen, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

[7] Robinson, H. W., Price, J. W., and Hogden, C. G., *J. Biol. Chem.*, 1937, **120**, 481.

[8] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1916, **24**, 117.

[9] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

[10] Folin, O., *J. Biol. Chem.*, 1933, **101**, 111.

[11] Young, E. G., and Conway, C. F., *J. Biol. Chem.*, 1942, **142**, 839.

[12] Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, **150**, 231.

time.

Pathology. The lesions were the same in all animals with progressive symptoms, whether they died spontaneously or were killed by exsanguination. They consisted in diffuse degeneration of the ganglion cells throughout the brain, without any visible difference between different areas. In Nissl sections, the ganglion cells showed either vacuolar degeneration, with swelling of the cell, disappearance of Nissl bodies and increased colorability of the dendrites, or pyknosis. Only occasional, normal cells were found. In Weigert-Pal sections, no definite changes could be found.

Clusters of unidentified cells were observed close to the ependymal lining. Such clusters have been described in various species. We are unable to decide whether these clusters have any pathological significance. No alterations were found in the liver, aside from moderate hyperemia of the capillaries. In none of our cases was there any fatty degeneration, such as has been described as one of the most constant features of DDT poisoning after ingestion of the drug.

No alterations were observed in other organs.

Conclusion. Progressive neurological symptoms were produced in cats by the intramuscular injection of DDT. The signs evolved in the sequence of stiffness, tremor, clonic movements, and death.

An animal with chronic neurological symptoms was not obtained, either because death supervened or because the animal returned toward normality.

Nissl sections revealed diffuse damage to the ganglion cells of the brain, characterized by vacuolar degeneration or pyknosis. No alterations, other than capillary dilatation, were recognized in the liver.

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Some Effects of Depletion and Repletion in Proteins on Body Fluids in Adult Dogs.*

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A decrease in plasma volume and a nutritional edema accompanies hypoproteinemia in dogs.[1-3] The fall in concentration of plasma proteins is associated also with an increase in the utilization of nitrogen, the nitrogen balance indexes of proteins being greater in protein-depleted than in normal animals.[4] An analysis of these data suggests that there are regular shifts in body fluids and in the utilization of nitrogen as the protein stores of the animal are altered. Experiments were organized, therefore, to study changes in plasma and "available fluid" volumes and in the excretion of various forms of nitrogen in adult dogs during control, depletion, and repletion periods.

Methods. Four dogs were depleted by feeding a protein-free diet; 3 days of plasmapheresis being used initially to speed up the process of depletion. The details of this method of

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1 Allison, J. B., Anderson, J. A., and Seeley, R. D., *Bull. N. Y. Acad. Sci.*, in press.

2 Weech, A. A., Goettsch, E., and Reeves, E. B., *J. Exp. Med.*, 1935, **61**, 299.

3 Weech, A. A., Wollstein, M., and Goettsch, E., *J. Clin. Inv.*, 1937, **16**, 719.

4 Allison, J. B., Seeley, R. D., Brown, J. H., and Anderson, J. A., *J. Nutrition*, 1946, **31**, 237.

TABLE II.

Daily Excretion of Compounds Containing Nitrogen During a 5-day Protein-free Feeding Period Before and After Depletion in Proteins and Repletion from Feeding for 35 Days 0.35 g of Hydrolysate Nitrogen per Day per Kilo of Body Weight.

Condition	Total N, g/day/sq.m.	Urea and Ammonia N, g/day/sq.m.	Creatine N, g/day/sq.m.	Creatinine N, g/day/sq m.	Uric acid N, g/day/sq.m.	Alantoin N, g/day/sq.m.	Amino N, g/day/sq.m.
			Lactalbumin Hydrolysate, Dog 28.				
Control	1.73	1.19	0.04	0.20	0.01	0.19	0.026
Depleted	0.84	0.46	0.02	0.15	0.01	0.19	0.031
Repleted	1.22	0.76	0.02	0.16	0.01	0.17	0.028
			Casein Hydrolysate, Dog 42.				
Control	1.35	0.84	0.03	0.17	0.01	0.24	—
Depleted	0.82	0.49	0.01	0.11	0.01	0.17	0.021
Repleted	1.60	1.06	0.04	0.14	0.01	0.20	0.022
			Casein Hydrolysate, Dog 68.				
Control	1.72	1.11	0.02	0.20	0.01	0.21	0.035
Depleted	1.13	0.70	0.02	0.16	0.02	0.16	0.025
Repleted	1.86	1.33	0.03	0.16	0.01	0.19	—

Fig. 3 illustrates data on urine nitrogen excretion obtained on 2 of the dogs, typical of all 4. The first point (C) records the urine nitrogen excretion of the normal dog while receiving the protein-free diet. The second point (D) records the excretion of urine nitrogen of the depleted dog while receiving the protein-free diet. The white squares and circles illustrate data obtained during the repletion process while dog No. 28 was receiving 0.35 g of lactalbumin hydrolysate nitrogen and dog No. 68 was receiving 0.35 g of casein hydrolysate nitrogen/day/kg body weight. The last black point records data obtained at 40 days, while the repleted dog was receiving a protein-free diet. These data prove that the excretion of body nitrogen on a protein-free diet is decreased below control values in the depleted dog. Upon repletion this excretion of body nitrogen returns toward control values, the body protein stores being replenished. The gradual increase in the excretion of nitrogen during the period of repletion is due to the gradual increase in body protein stores accompanied by a decrease in retention of dietary nitrogen. These data supplement those previously published where a reduction from control values in nitrogen excretion and an increase in nitrogen balance index of the dietary protein above normal was demonstrated in the depleted dog.[4]

Table II records the average daily urine excretion of compounds containing nitrogen during the protein-free feeding periods before and after depletion and after repletion in proteins.

These data prove that the decrease below control levels of urine nitrogen in the depleted dog is due primarily to a decrease in ammonia and urea nitrogen.

Summary. The plasma albumin decreases markedly while the total plasma globulins decrease slightly below control values in the protein-depleted animal. The dogs are very susceptible to disease in the depleted state, a susceptibility which is reduced upon repletion in proteins. The plasma volume drops and the available fluids increase as the total circulating plasma albumin is reduced by the process of depletion. This nutritional edema is corrected rapidly by repletion in plasma albumin.

The excretion of urine nitrogen gradually increases during the feeding of protein nitrogen in the repletion period. This gradual increase is the result of a decrease in the utilization of the protein and of an increase in the excretion of nitrogen from body stores. The decrease in urine nitrogen excretion below normal which occurs in the hypoproteinemic dog is primarily the result of a decrease in ammonia and urea nitrogen.

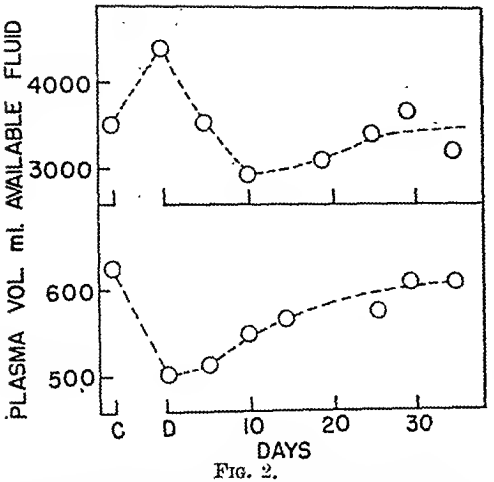

FIG. 2.
Available fluid and plasma volumes before (C) and after depletion in proteins (D) and after 35 days of feeding 0.35 g of casein hydrolysate nitrogen/day/kilo of body weight.

effect of depletion upon plasma proteins in one dog, typical of all 4. These data prove that plasma albumin decreases markedly while plasma globulins decrease slightly below control values in the depleted animal. The reduction in globulin fractions may represent a decrease in the protein stores of the lymphatic tissues. Certainly the α globulin fraction is not reduced, the reduction in globulins being restricted to the β and γ fractions.[13] Under these conditions, the dogs become very susceptible to disease, developing kennel sores and other skin disorders. Replenishment of protein stores by feeding a high-quality protein restores rapidly the natural resistance of the animal to these disturbances. Fig. 1 demonstrates the gradual return of albumin and globulin fractions from the depleted to the normal condition when the dog was fed a casein hydrolysate. Similar regenerations were found in the other 3 animals, one receiving the casein hydrolysate and the other 2 lactalbumin hydrolysate. A critical evaluation of the regeneration of the plasma proteins in these experiments has been made through the analysis of electrophoretic patterns which were obtained on these and a number of other dogs.[14]

[13] Chow, B. F., Allison, J. B., Cole, W. H., and Seeley, R. D., PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 14.

The data in Table I record the effects of depletion and repletion in proteins on the plasma and available fluid volumes.

The plasma volume decreases below the control value in the depleted dog, returning toward normal during the repletion period. The available fluid, on the other hand, increases in the protein-depleted dog, returning to control levels upon repletion. Thus the ratio between the available fluid and plasma volumes increases in the depleted animals to above normal. This increased ratio detects a "nutritional edema" long before increased fluid in tissue spaces can be observed clinically.

The data in Fig. 2 illustrate graphically, in more detail than in Table I, the changes in available fluid and plasma volumes which accompany depletion and repletion in proteins. The rapid decrease of available fluid volume which occurred in all dogs soon after the repletion process started is especially noteworthy.

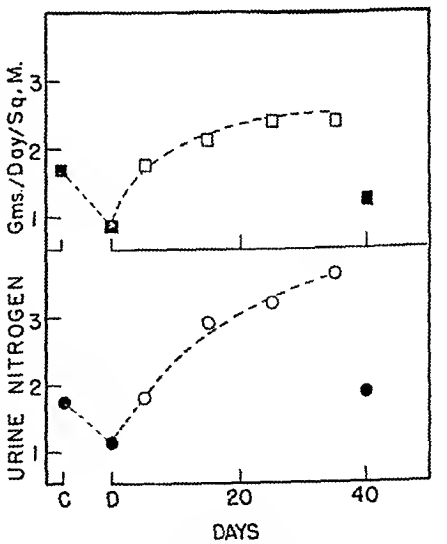

FIG. 3.
Total daily urine nitrogen excretion in dogs 28 □ and 68 ○ while on a protein-free diet before (C) and after depletion in proteins (D) and during 38 days of feeding 0.35 g of hydrolysate nitrogen/day/kilo of body weight. The last point at 40 days represents the daily excretion of nitrogen on a protein-free diet after repletion.

[14] Chow, B. F., Allison, J. B., Cole, W. H., and Seeley, R. D., in press.

treatment with neostibosan is small (3 out of 11 examined or 27.3%) there is evidence indicating that the drug has parasitotropic effects. This is also suggested by the fact than the egg count 11 months after treatment in 6 of the patients are appreciably lower than the counts before treatment. There is the possibility that in those patients in whom the infection was not eradicated insufficient drug was administered. Retreatment with neostibosan was found to be necessary by Culbertson, Rose and Oliver-González in humans infected with microfilaria of *W. bancrofti*.[2] It seems possible that the use of a greater amount of drug may produce a larger percentage of cures.

[2] Culbertson, J. T., Rose, H. M., and Oliver-González, J., *Am. J. Hyg.*, 1946, **43**, 145.

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Treatment of *Schistosomiasis mansoni* with Neostibosan.*

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Twelve patients with ova of *S. mansoni* in their stools were treated with the pentavalent antimonial drug, neostibosan (Winthrop). The patients were all males with ages ranging from 15 to 32 years. The drug was administered intravenously during a 2-week period of hospitalization. During the first 3 days, doses of 0.2, 0.6 and 0.9 respectively were usually given, and thereafter either 0.6 or 0.9 was generally injected every day until the end of hospitalization. All patients tolerated the drug well and no serious reactions were seen. A low grade fever and abdominal pain, headache, nausea or anorexia were observed in most of the patients.

The effect of the drug on the number of schistosome eggs passed in stools was carefully observed. Stool examinations were made daily before and during treatment and on as many daily specimens as possible for each follow-up examination. The technic used for the quantitative determination of eggs in feces has been described previously.[1]

The effect of treatment on the schistosome infections, as determined by the number of alive and dead schistosome ova in stools may be seen in Table I. Although the majority of the patients (9 out of 12 or 75%) had stools negative for live and dead ova one to 2 months after treatment, 8 out of 11 (73%) relapsed 3 and 4 months later. Three out of 11 patients, however, have remained negative throughout, for a period of 11 months.

Although the per cent of cases which has remained negative for ova of *S. mansoni* after

TABLE I.
Number of Live and Dead Schistosome Eggs in Patients Treated with Neostibosan.

Total drug administered	Eggs per ml of stool: Before treatment		Months after treatment: 1		2		5		6		8		11	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
9.5	780	2,730			585	2,145			195	195			195	0
8.6	780	3,900			195	780			1,365	390	1,170	1,050		
8.9	1,560	1,755			0	0			585	1,365	5,070	3,510	390	780
8.6	7,020	3,900			2,340	1,950			390	585	1,170	1,365	1,170	488
7.7	1,950	585			0	0			95	0	0	0	195	585
9.5	1,560	1,365			0	0			0	195	0	0	195	195
12.5	975	585	0	0	0	0	0	0			0	0	0	0
11.6	195	2,340	0	0			0	0			0	0	0	0
12.2	2,340	1,170	0	0							0	0	98	0
12.5	1,755	585	0	0	0	95	390	195			0	98	0	0
12.5	1,365	3,315	0	0	0	0	0	0			0	0	0	0
8.9	390	195	0	0	0	390	0	95			0	98	0	0

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the School of Tropical Medicine, San Juan, P.R.

1 Hernández-Morales, F., Suárez, Ramón M., Pratt, C. K., and Oliver-González, J., *Puerto Rico J. Pub. Health and Trop. Med.*, June, 1946.